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AGE AND SEX DIFFERENCE IN SERUM AND PITUITARY THYROTROPIN CONCENTRATIONS IN THE RAT: INFLUENCE BY PITUITARY ADENOMA

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Abstract—Effects of age and sex difference on serum and pituitary thyrotropin (TSH) concentrations were investigated in young (4 to 6 months) and old (28 to 36 months) Long-Evans rats with or without pituitary tumor. Basal and TRH-induced serum TSH concentrations in young males are significantly ($P < .05$) higher than in old males, while there is no significant difference in basal TSH between young and old female rats, but young females had greater TSH response to TRH than the old females. Orchidectomy significantly reduced plasma TSH in the young but not in the old male rats. Administration of testosterone propionate reversed the castration effect on TSH in the young male rat. Ovariectomy significantly reduced plasma TSH in the old but not in the young female rats. Estrogen treatment slightly increased basal TSH concentrations in both the young and old rats.

Castration of both male and female reduced pituitary TSH response to TRH and was reversible in young but not old rats. Young male rats had significantly higher concentrations of basal and TRH-induced TSH release than their female counterparts.

The pituitary TSH content of young but not old male rats was significantly higher than their female counterparts of the same age. Pituitary TSH content of old animals with pituitary adenoma was significantly lower than those without pituitary tumor. Since 39% of the old male and 75% of the old female rats had pituitary tumor, a difference in pituitary responsiveness to various physiological and pharmacological stimulation may be partially due to difference in pituitary tumor incidence.

INTRODUCTION

AGE HAS been shown to be a factor in the function of hypothalamic pituitary-thyroid axis in rats. Both earlier and recent studies have been reviewed (Gregerman and Bierman, 1981). In old rats, the rate of thyroxine (T_4) secretion decreases by 50% (Grad and Hoffman, 1955). Although T_4 distribution space and metabolic clearance increase (Frolkis and Valueva, 1978), thyroid function decreases with age (Narang and Turner, 1966). Thyrotropin release in response to thyrotropin releasing hormone (TRH) stimulation may differ according to estrogen or androgen treatment in female (Chen and Walfish, 1978a) and male (Chen and Walfish, 1979) rats of Long-Evans strain. In the Sprague-Dawley male rats TSH response to TRH is reported to have no difference between young and old rats (Flug and Adelman, 1979). In humans, pituitary thyrotropin response to TRH

decreases in men (Snyder and Utiger, 1972a) but not in women (Snyder and Utiger, 1972b).

Sex-related differences in thyroid function have long been documented. However, results are controversial. Several reports show that female rats had elevated TSH concentrations when compared with adult males (Rap and Pyun, 1974; Kieffer *et al.*, 1976; Simpkins *et al.*, 1976). Sex steroid increased TSH response to TRH (Chen and Walfish, 1978a,b; Kieffer *et al.*, 1976), while administration of estrogen increased serum T_3 and decreased serum T_4 concentrations in female rats (Chen and Walfish, 1978b). By contrast, in a recent study testosterone has been shown to be responsible for higher titer of thyrotropin in the male than in the female rat (Christianson *et al.*, 1981).

Although both age and sex effect on TSH release has been studied, no study has been reported to compare thyrotropin release in aged male and female rats. The purpose of the present investigation was to compare thyrotropin release in intact, castrated, or castrated sex-steroid-replaced young and old, male and female rats, with particular attention to the presence of absence of pituitary adenoma of the old rats.

MATERIALS AND METHODS

Male and female rats of the Long-Evans strain were obtained from Blue Spruce Animal Farm, Charles Rivers, Farmington, New York, U.S.A. They were obtained as retired breeders at 8 to 12 months of age and were housed at the Vivarium of Barrow Neurological Institute, St. Joseph's Hospital and Medical Center. Animals were used for experiments until they were 28 to 30 months of age. The body weight of old male rats ranged from 800 to 1100 g whereas the body weight of intact female rats ranged from 260 to 300 g.

Some animals were castrated for at least one month before they were used for experiments. Male and female animals of 4 to 6 months of age of the same strain were used as controls. These intact animals were sham-operated.

Old and young, intact or castrated, rats were treated with 0.1 ml corn oil (controls) or, in the case of castrated animals, 2 ug estradiol benzoate (E_2) or 2 mg testosterone propionate (TP) in 0.1 ml corn oil. All treatments were for a period of seven days. On the day following the last injection of the steroids, one blood sample was obtained as basal level of TSH.

Injection of thyrotropin-releasing hormone

One day after the last injection of corn oil, E_2, or TP, a basal blood sample was collected from all animals before they were given a single s.c. injection of 100 ng TRH/100 g BW. 15 minutes after TRH injection, another blood sample was collected. Blood samples were obtained via puncture of the ocular sinus under light ether anesthesia as prescribed previously (Chen and Walfish, 1979) or, in the case of the animals killed at the end of the experiment, blood was collected from the trunk after decapitation to examine for the existence of pituitary adenoma. Blood samples were allowed to stand at room temperature for 2 hours before they were placed in a refrigerator to stand for about 12 hours before they were centrifuged. Serum obtained from these blood samples was stored at $-20°$ C until assay for TSH concentration.

Collection and homogenization of pituitary and pituitary adenoma

Two to three weeks after the animals were last treated, all young and old animals were killed by decapitation. Pituitary glands from both young and old rats were dissected from the sella and weighed. In the case of the old rats, the pituitary gland was dissected for examination of the pituitary under a biopsy microscope. When a pituitary adenoma was confirmed, a portion of the tumorous tissue was dissected, weighed and treated as pituitary glands of the young animals.

After the pituitary or pituitary adenoma tissues were dissected and weighed, they were placed in a test tube containing 1% bovine serum albumin phosphate buffer saline (BSA-PBS), in the concentrations of 1 mg/ml. The pituitary and pituitary tumor were homogenized in ice with a sonicator.

Radioimmunoassay of thyrotropin

Radioimmunoassay for serum and pituitary TSH was performed using a radioimmunoassay kit generously supplied by the National Pituitary Agency, NIAMDD. The reference used was Rat-TSH-Reference-1. Pituitary

and pituitary adenoma homogenates were further diluted to 1:20 V/V with 1% BSA-PBS. 50 to 100 ul of the diluted samples were assayed for TSH concentrations.

Analysis of data

Student's *t*-test was used to compare data from two independent groups; a paired test was used when comparison within a group with its own pretreatment value was made, as was the case after TRH stimulation.

RESULTS

Table 1 summarizes the effects of castration and replacement of estradiol benzoate or testosterone propionate on basal and TRH-induced TSH release in rats. Intact young male rats had significantly ($P < 0.05$) higher serum TSH concentrations than intact old male and intact young female rats. Castration decreased TSH concentration in young but

TABLE 1. EFFECT OF AGING, CASTRATION AND GONADAL STEROIDS ON BASAL AND TRH-STIMULATED TSH CONCENTRATIONS IN LONG-EVANS RATS

Age	*Group*	*Treatment*	*n*	*TSH concentration (ng/ml)* basal	*TRH-stimulated*
Young male (4–6 months)	1	Intact	8	724 ± 83	5085 ± 511
		P1–2		< 0.001	< 0.01
	2	Orchx	6	307 ± 78	3259 ± 695
		P2–3		< 0.001	N.S.
		P1–3		N.S.	N.S.
	3	Orchx + TP	8	905 ± 135	4161 ± 633
Old male (28–36 months)	4	Intact	6	446 ± 69	3621 ± 545
		P1–2		< 0.01	< 0.05
		P4–5		N.S.	< 0.02
	5	Orchx	4	460 ± 58	2726 ± 188
		P5–6		N.S.	N.S.
		P6–7		N.S.	N.S.
	6	Orchx + TP	4	598 ± 81	2554 ± 744
Young female (4–6 months)	7	Intact	9	362 ± 77	3260 ± 221
		P1–7		< 0.001	< 0.05
		P7–8		N.S.	< 0.02
	8	Ovx	6	318 ± 50	2024 ± 319
		P8–9		N.S.	N.S.
		P7–9		N.S.	N.S.
	9	Ovx + E_2	8	451 ± 56	3537 ± 452
Old female (28–36 months)	10	Intact	5	540 ± 125	1759 ± 193
		P4–10		N.S.	< 0.05
		P10–11		< 0.01	N.S.
	11	Ovx	5	201 ± 35	1103 ± 250
		P11–12		N.S.	N.S.
		P10–12		< 0.02	< 0.01
	12	Ovx + E_2	5	247 ± 24	820 ± 107

Abbreviations: TP – testosterone propionate
Orchx – orchidectomy
Ovx – ovariectomy
E_2 – estradiol benzoate

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TABLE 2. EFFECTS OF AGING ON PITUITARY ADENOMA INCIDENCE IN MALE AND FEMALE RATS.

		Tumor Incidence	*%*
Young	Male	0/11	0%
	Female	0/12	0%
Old	Male	7/18	39%
	Female	12/16	75%

not in old male rats. TP treatment also restored TSH to intact levels in young rats. TSH response to TRH was significantly higher in young intact male rats than in castrated young or intact old male rats. The decrease in TSH to TRH in castrated young but not in old male rats was restored after TP treatment. No significant difference in basal serum TSH concentration was observed between young and old intact female rats. Castration decreased serum TSH in the old but not young female rats. TRH-induced TSH release was significantly greater in young than in old female rats regardless of surgical or E_2 treatment. Estrogen treatment slightly increased TSH response to TRH in the young but not in the old female rat.

Table 2 shows the incidence of pituitary adenoma in intact young and old rats. No pituitary adenoma was observed in intact male or female rats of age up to six months old. However, in animals between 26 to 30 months of age as many as 39% of the male and 75% of the female rats had pituitary adenoma. Some pituitary tumors reached a diameter of 5 to 7 mm.

TABLE 3. EFFECTS OF AGING AND PITUITARY TUMOR ON THYROTROPIN SERUM CONCENTRATIONS IN MALE AND FEMALE RATS (BLOOD SAMPLES COLLECTED BY DECAPITATION).

		n	*TSH (ng/ml)*	*P*
Young	Male	13	1195 ± 83	p< 0.01
	Female	12	435 ± 40	
Old	Male (28-36 months) (without tumor)	9	821 ± 119	p< 0.05
	Female (28-36 months) (without tumor)	10	413 ± 64	
Old	Male (with tumor)	6	553 ± 56	N.S.
	Female (with tumor)	12	503 ± 62	

TABLE 4. EFFECTS OF AGING AND PITUITARY ADENOMA ON PITUITARY THYROTROPIN CONCENTRATIONS IN THE RAT.

			ug/mg pituitary wet weight	*P*
Young	Male	13	133 ± 5	$p < 0.01$
	Female	12	106 ± 6	
Old (without tumor)	Male	6	114 ± 21	N.S.
	Female	4	104 ± 12	
Old (with tumor)	Male	4	53 ± 14*	N.S.
	Female	8	42 ± 14*	

All values are expressed as means ± SEM.
*$p < 0.001$ when compared with either young or old rats without pituitary tumor.

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Table 3 shows pituitary and serum TSH concentrations in male and female rats with or without pituitary adenoma. The serum was obtained from trunk blood collected after decapitation. Young males had significantly higher serum TSH concentrations than old males or young females. Old male rats also had greater serum TSH concentrations than old females. This sex-dependent difference in serum TSH was not seen in the old rat bearing pituitary adenoma.

Table 4 shows pituitary TSH content in young and old rats with or without pituitary tumor. Young male pituitary also contained greater amounts of TSH than those of young female or old male animals. This difference, however, was not statistically significant because of greater variability. Pituitary TSH content of old male and female rats bearing pituitary tumors was significantly lower than that in rats of the same age without pituitary tumors.

DISCUSSION

The finding that TSH-response to TRH was reduced in old but not young rats was in agreement with previous reports by Chen and Walfish (1978a, 1979), but was in disagreement with another report (Klug and Adelman, 1979). However, in the latter report Sprague-Dawley instead of Long-Evans rats were used. This study confirmed our previous report (Chen and Walfish, 1979) that TSH response to TRH of the old rat did not increase after TP treatment. Since testosterone was shown to be responsible for elevation of circulating TSH concentrations (Christianson *et al.*, 1981), this may help to explain why orchidectomy decreased basal TSH concentration in the young, which had higher circulating testosterone, but not in the old male rats, which already have decreased circulating testosterone (Chen and Walfish, 1979). Thus, further decrease in testosterone by castration in the old male rat might not cause further decrease in serum TSH.

One of the reasons why the TSH response to TRH decreased in the old but not in the young rats, regardless of sex, must be due to the presence of pituitary adenoma. The incidence of pituitary adenoma may explain why treatment with TP or E_2 did not restore TRH-induced TSH release in the aged male or female rats. In this study, 39% of the males

and 75% of the females had pituitary adenoma at autopsy. The high percentage of pituitary tumors may be due to the relative senility of the rats used. Few, if any, previously published studies on pituitary responsiveness to LH-RH or TRH involved killing of the animal for determination of the presence of pituitary adenoma. In view of the findings in the present experiment, the study of effects of aging on pituitary function must take into account the possible presence of pituitary tumors. Therefore, it is not only discrete but also necessary to verify the presence or absence of pituitary adenoma when studying pituitary responsiveness in aging Long-Evans rats 24 months and older.

The observation that male rats had significantly higher circulating basal and TRH-stimulated TSH concentrations is in agreement with the report by Christianson *et al.* (1981) who showed that testosterone was responsible for the increase in TSH in rats. In both the old and young male rats without pituitary adenoma, serum TSH concentrations were significantly higher in the male than in the female. However, no such difference was observed in animals with pituitary adenoma (Table 2). This is evidence that pituitary adenoma masked the effect of sex steroid on TSH concentrations.

Although Chen and Walfish (1978a) reported that there was no difference in TRH-induced TSH release in intact young and old female rats, the present experiment indicated that the older female (28 to 36 months) had significantly diminished TSH response to TRH than the young female rats. Furthermore, E_2 treatment restored TSH response to TRH in the young but not in the old rats, suggesting that high incidence of pituitary tumors may be responsible for the diminished TSH response to TRH in the old female rats.

In conclusion, these data indicate that the older the animal, the greater the incidence of pituitary adenoma in this strain of rat. The study of hormone change during the aging process is greatly affected by the presence or absence of pituitary tumor. This influence by pituitary tumor should be taken into consideration for not only TSH, as in the present study, but also for other pituitary hormones including LH, prolactin, FSH, GH, ACTH, and perhaps beta-endorphin and their target organs.

Acknowledgements—The author wishes to thank Dr. A. Parlow and the National Pituitary Agency, U.S.A. for supplying a TSH RIA kit, and Ms. Deirdre Anne Janus for her secretarial assistance. This study was supported in part in NIH BRSG grant #S07-RR-05872.

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EFFECT OF 2-MERCAPTOETHYLAMINE ON PROLIFERATION AND LIFESPAN OF WI-38 CELLS

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Abstract—The antioxidant 2-mercaptoethylamine (MEA) was added to the culture medium of WI-38 cells throughout their lifespan. MEA concentrations of 0.5μM, 5μM and 50μM were used. The presence of 5μM or 50μM MEA decreased the fraction of rapidly dividing cells and slightly reduced cell density at confluence, whereas 0.5μM MEA had no significant effect on these parameters. All MEA-treated cultures had somewhat reduced plating efficiency. Cultures treated with 0.5μM, 5μM and 50μM MEA attained population doubling levels of 72.80, 78.35 and 70.92, respectively, compared to 73.55 for untreated controls. Cells grown in 5μM MEA underwent 4.8 more population doublings, in six weeks more time in culture, compared to controls. This is a 12.2% increase in the portion of the lifespan examined and a 6.5% increase in total lifespan. These results are consistent with the free radical theory of aging.

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INTRODUCTION

ACCUMULATION OF damage through free radical reactions has been postulated to be a major cause of the aging process (see review by Harman, 1981). One way of testing this hypothesis is to determine the effect on lifespan of substances (such as antioxidants) which inhibit the propagation of free radical reactions (Harman, 1968). While a number of studies have been carried out with this aim, results have been inconclusive. Median lifespan and maximum lifespan have been extended beyond those of controls in many cases (Harman, 1968; Kohn, 1971; Epstein and Gershon, 1972; Munkres and Minssen, 1976; Miquel and Economos, 1979) but a lengthening of maximum lifespan beyond the optimum value reported for the species has not been attained (Kohn, 1971; Harman, 1981). This last observation is crucial to evaluating a true retardation of the aging process. Some of the greatest increases in lifespan have been obtained with the antioxidant 2-mercaptoethylamine (MEA). Addition of 1% MEA to the diet of LAF_1 mice increases their mean lifespan by 30% (Harman, 1968). Kohn (1971) increased the mean and maximum lifespan of the long-lived C57BL/6 mouse strain by the addition of 1% MEA to the diet, but in no case did the increase exceed the optimum lifespan for the strain. One confounding factor in these studies is that addition of antioxidants to the diet can reduce body weight and presumably food consumption (Harman, 1968). Since it is well known

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that food restriction can increase lifespan, it is difficult to separate this effect from one due to suppression of free radical reactions. One way of circumventing this difficulty is to use an *in vitro* model of aging. While the relationship between aging of serially-subcultivated cells in tissue culture and aging *in vivo* is not completely understood, there is little doubt that *in vitro* aging is a relevant and important model (see reviews by Hayflick, 1980; Cristofalo and Stanulis-Praeger, 1982). The purpose of this study was to examine the effect of MEA on proliferation and lifespan of the fetal lung fibroblast-derived human diploid cell strain, WI-38. It is reported here that MEA in the culture medium generally slowed cellular proliferation; cells grown in 5μM MEA had a longer calendar lifespan and underwent more population doublings compared to controls.

METHODS AND PROCEDURE

WI-38 cells were obtained from frozen stocks at the Wistar Institute, Philadelphia, PA. Cell cultures were maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum (both purchased from Flow Laboratories) as described by Cristofalo and Charpentier (1980). Briefly, cultures were subcultivated weekly (if confluent) in 75-cm^2 tissue culture flasks with a constant seeding density of 7.5×10^5 cells in 40ml of medium (10^4 cells/cm^2 growth surface; 0.53ml medium/cm^2 growth surface). Cells were at population doubling level (PDL) 34.2 at the start of the experiment. The PDL was calculated at each subcultivation based on cell counts. When cultures no longer reached confluence within a week, the medium was replaced with fresh medium. If after three weeks of refeeding the culture was still not confluent it was harvested anyway and counted. If the cell number had not doubled in that time the culture was considered to be phased out. Judged by these criteria most WI-38 sublines phase out at PDL 65 to 75 (Cristofalo and Charpentier, 1980).

MEA (Sigma) was made up in a stock concentration in sterile medium and added to the culture medium at the time of subcultivation to obtain final concentrations of 0.5μM, 5μM, and 50μM. A control culture had no MEA added. Preliminary experiments showed that MEA concentrations greater than 100 μM drastically inhibited cell growth.

Growth curves were generated during the sixth week of the experiment by seeding cells into multiwell plates at the same cell/area and volume/area ratios used for flasks. Samples were counted in triplicate 1, 2, 4, 6, 8, 10 and 12 days after seeding.

After six weeks of MEA treatment the percentage of cells incorporating thymidine was determined by incubating cultures one day after seeding with ^{3}H-thymidine for 30 hours. Following autoradiography the percentage of cells with grains above the nucleus was counted. The percentage of labeled nuclei (PLN) determined in this manner is a measure of the percentage of cells entering DNA synthesis and declines as a culture ages (Cristofalo and Sharf, 1973).

RESULTS

The presence of 5μM or 50μM MEA in the growth medium resulted in slightly lower cell density at confluence (Figure 1) and slowed cellular proliferation. The decreased fraction of these MEA-treated cells entering DNA synthesis, as indicated by the reduction in percent labeled nuclei (Table 1), correlates with this observation. The addition of 0.5μM MEA had very little, if any, effect on these parameters. All MEA-treated cultures had a slightly reduced plating efficiency, that is, fewer cells attached to the growth surface on the first day after seeding (Figure 1).

Control culture phased out at PDL 73.55 (Figure 2). Cells cultured with 0.5μM and 50μM MEA phased out at PDLs of 72.80 and 70.92, respectively, at the same chronological age as the control. Cells cultured with 5μM MEA phased out at PDL 78.35, six weeks later than controls and others.

DISCUSSION

These results indicate that MEA can produce a small but definite extension of the lifespan of WI-38 cells, as measured both by number of population doublings and calendar

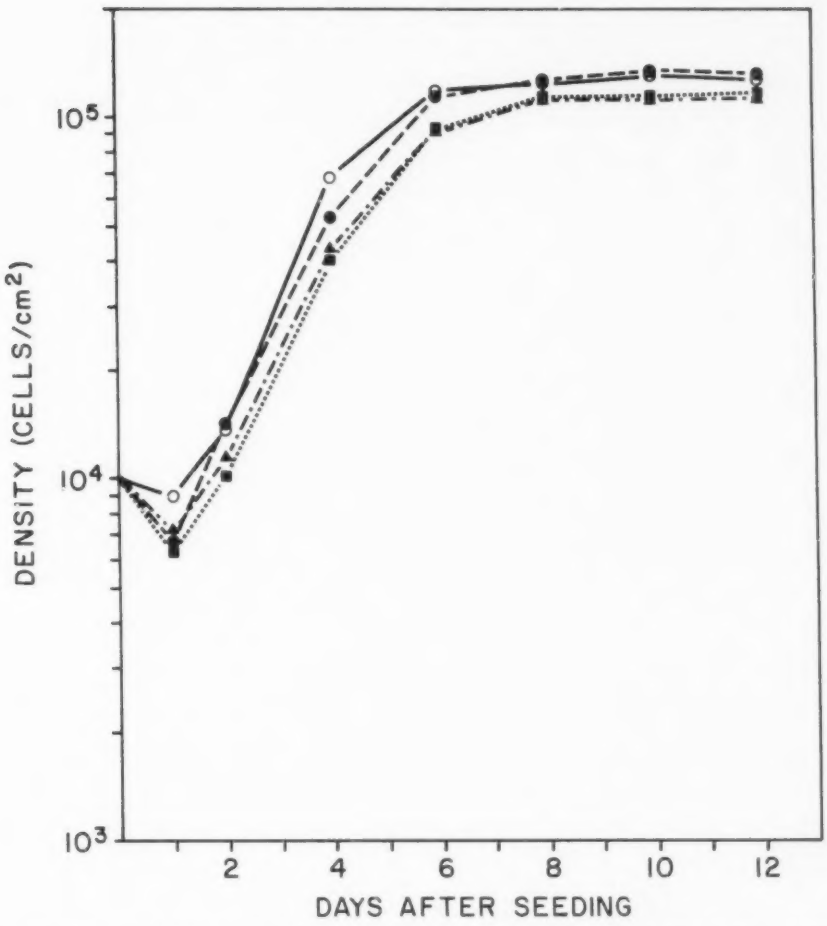

FIG. 1. Growth curves of MEA-treated cells and controls. Cell density (cells per cm² of growth surface) is plotted against time, all cultures having been seeded at 10^4 cells/cm² at time zero. Control ○——○; 0.5μM MEA ●– – – –●; 5μM MEA ■----------■; 50μM MEA ▲-·-·-·-·-▲.

TABLE 1. PERCENT LABELED NUCLEI OF CONTROL AND MEA-TREATED CELLS AFTER SIX WEEKS IN CULTURE

	PDL	*Percent Labeled Nuclei*
Control	56.4	90
0.5μM MEA	56.2	87
5μM MEA	55.2	82
50μM MEA	54.4	71

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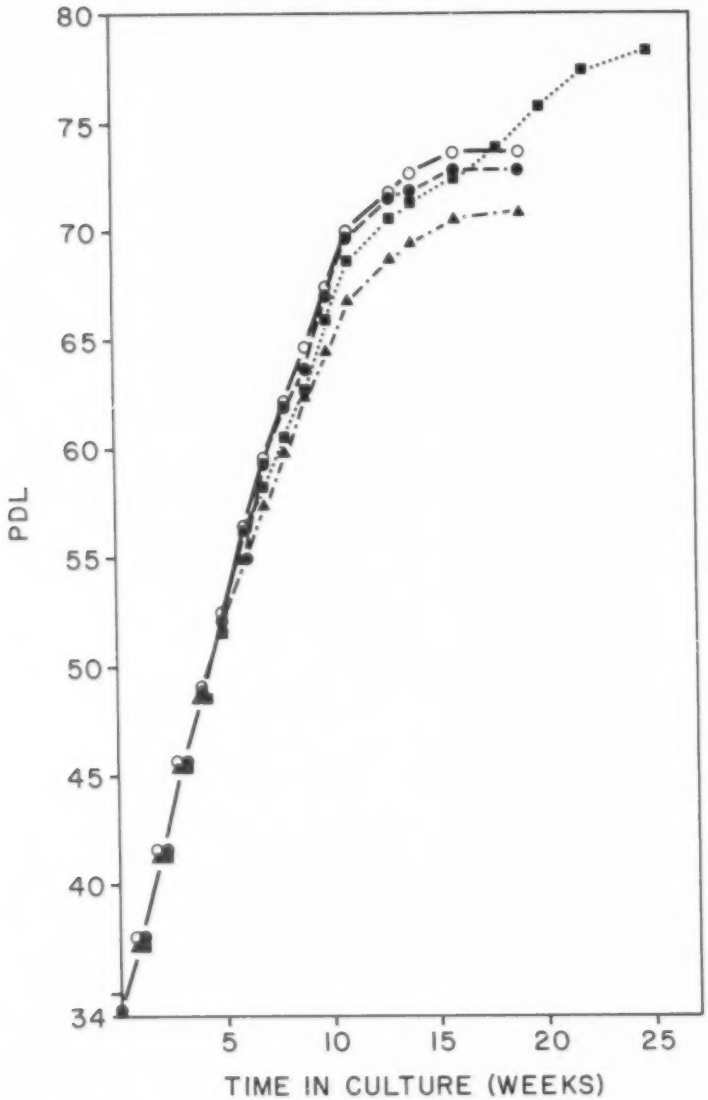

FIG. 2. Population doubling level of MEA-treated cells and control plotted against time. Control ○ —— ○; 0.5μM MEA ● – – – – ●; 5μM MEA ■----------■; 50μM MEA ▲-·-·-·-·-▲.

time. It is thought that the number of population doublings, rather than time in culture, is the main parameter determining *in vitro* lifespan of a culture (Dell'Orco *et al.*, 1974; Cristofalo and Stanulis-Praeger, 1982). This effect was dose-dependent. The presence of 5μM MEA resulted in 4.8 more population doublings than control. This amounts to a 12.2% increase in the portion of the lifespan examined in this study (PDL 34.21 to PDL 73.55), or a 6.5% increase in total lifespan. A concentration of 0.5μM MEA had essentially

no effect and 50μM MEA caused a slightly decreased lifespan. The reduced plating efficiency seen in the MEA-treated cultures may well have detracted from other, beneficial effects of the compound. It is possible, of course, that the concentration of MEA could be optimized further than in this study, in which the concentrations used were separated by an order of magnitude.

It is interesting to note that MEA did not cause any increase in cell saturation density or in the fraction of rapidly proliferating cells. In fact, 5μM and 50μm MEA decreased both. Hydrocortisone, which also extends the lifespan of WI-38 cells, increases these parameters (Cristofalo and Kabakjian, 1975; Cristofalo and Stanulis-Praeger, 1982).

The findings of this study are consistent with the free radical theory of aging. A more dramatic increase in lifespan may require combinations of antioxidants capable of penetrating the various cellular compartments. While initial reports indicated that the natural antioxidant vitamin E could significantly extend *in vitro* lifespan, subsequent studies found that addition of vitamin E to the culture produced no increase (Balin *et al.*, 1977; Packer and Smith, 1977). One reasonable approach to this question would be the administration of combinations of antioxidants (for example, MEA, vitamin E, selenium) in various concentrations to a relatively short-lived model such as rotifers or chick cells in culture, to rapidly assess the maximum life-extending capability of known substances. In particular, Harman (1981) has suggested that antioxidants would need to penetrate the mitochrondrial membrane, in which most oxygen is utilized, to have significant anti-aging effects. In terms of cellular aging, however, there is evidence that the process is controlled mainly by the nucleus (Hayflick, 1980).

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INDIVIDUAL ANALYSIS OF AGE-ASSOCIATED CHANGES IN REPRODUCTIVE ACTIVITY AND LIFESPAN OF *DROSOPHILA VIRILIS*

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INTRODUCTION

MANY STUDIES on the effects of genetic and environmental factors on *Drosophila* lifespan have been conducted (Pearl, 1928; Clarke and Maynard-Smith, 1961; Maynard-Smith, 1963; Kidwell and Malick, 1967; Lamb, 1968; see also reviews by Lints, 1971; Lamb, 1978). At the population level, extensive variations in lifespan has been reported even in such genetically homogeneous populations as highly inbred laboratory strains. Undoubtedly, various environmental factors play important roles in determining the lifespan of *Drosophila*. Besides temperature and culture media factors, which have great effects on the lifespan of *Drosophila*, reproductive conditions may also exert an influence.

There have been some papers which reported the relationship between reproductive activity and lifespan in *Drosophila*. *D. melanogaster* females, sterilized by ionizing radiation (Lamb, 1964) or by heat treatment (Maynard-Smith, 1958), did not lay as many eggs as non-treated control females and lived longer than controls. The lifespan of virgin *melanogaster* males was longer than that of mated males (Bilewicz, 1953). Sterile XO

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males lived longer than normal XY males (Tobari and Moriwaki, 1961). These results suggest a negative relationship between reproductive activity and lifespan; that is, low reproductive activity lengthens lifespan and high activity results in a reduction in lifespan.

On the other hand, there are other data which indicate that reproductive activity does not correlate with lifespan in the simple ways described above. In *D. virilis,* virgin females always lived longer than mated females even though there was no significant difference in egg-laying activity between these two groups. Also, the lifespan of virgin unmated *D. virilis* males was shorter than that of mated males. In addition, the lifespan of *virilis* was affected by both the sex ratio and population density (Aigaki and Ohba, 1983).

It is clear from the above reports that reproductive activity plays an important role in determining the lifespan of *Drosophila* and that further detailed analyses are necessary in order to understand the causal relationship between reproductive activity and lifespan. For this purpose both age-associated changes in reproductive activity and survivorship were individually examined in *Drosophila virilis* throughout their lifespan. The results of this study are reported in this paper.

MATERIALS AND METHODS

Flies

The TK strain of *Drosophila virilis,* a highly inbred laboratory strain, was used in all experiments. Flies were collected within 12 hours of the 14th day after oviposition and kept individually in vials (30 × 105 mm) with culture media. Culture vials were renewed three times a week and survivorship was checked each day throughout the lifespan. The age of flies were expressed in either days or weeks. All experiments were carried out at 25 ± 0.5°C under constant lighting of 100–200 lux.

Culture media

For raising flies the standard yeast medium I, the same medium as that used in maintaining stock cultures, was chosen. For aging studies flies were fed on the standard medium III. Details of both media are shown in another paper (Aigaki and Ohba, 1983).

Measurement of male reproductive activity

Each individual male fly was placed in an empty vial containing three ten-day old virgin female flies. After an hour the male was returned to its culture vial and the three females were individually separated into three new culture vials. After 24 hours each female was examined for the presence of sperm in her reproductive organs. The number of females in which sperm was found was regarded as an indication of the number of copulations during the one hour test period. In addition, the number of eggs deposited by individual copulated females during the 24 hour period and the number of flies which emerged from these eggs were also recorded.

Measurement of female reproductive activity

Female flies were tested in a manner similar to that described for males. Each female fly was placed in a vial together with three ten-day old unmated male flies. The number of copulations in a one hour period was observed and recorded. Then the female was transferred to a new culture vial for a 24 hour period. The number of eggs deposited during that period as well as the number of flies emerging from these eggs were noted.

Measurements of male and female reproductive activity were made at one week intervals and always at the hours of 14:00 to 15:00 for males and from 16:00 to 17:00 for females.

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RESULTS

Age-associated changes in male reproductive activity

Mating activity. Figure 1 shows the weekly changes in the proportion of male flies which copulated once, twice or three times in each one hour test period. In the first and second tests, at the age of one and two weeks after eclosion, more than 90% of flies copulated at least once. At five weeks of age the proportion of copulating males was more than 70%. This proportion then decreased rapidly falling to 0% at the twelfth week. This reduction in male copulating activity preceded the decrease in the number of surviving flies. With the exception of the results at three weeks of age, the proportion of non-copulating males increased gradually with age until 12 weeks. After that time it remained 100% until all males had died 19 weeks after eclosion.

The proportion of flies which copulate three times in the one hour period may be the best indicator of male mating activity. This proportion had a single peak at two weeks of age where about one half of the males copulated three times during the short period. The proportion then decreased rapidly, arriving at a level between 10 and 15% at four weeks of age. Few males older than eight weeks copulated three times under the given test conditions. The proportion of males which copulated twice exhibited a peak at one week and another at four weeks of age. No clear peak was observed for the proportion of males mating once, though it was relatively high at one, five, eight and nine weeks.

Age-associated changes in male mating activity is clearly seen in Table 1 in which the mean number of copulations per surviving males is shown together with the standard error and coefficient of variation. Male copulating activity was high during the first four

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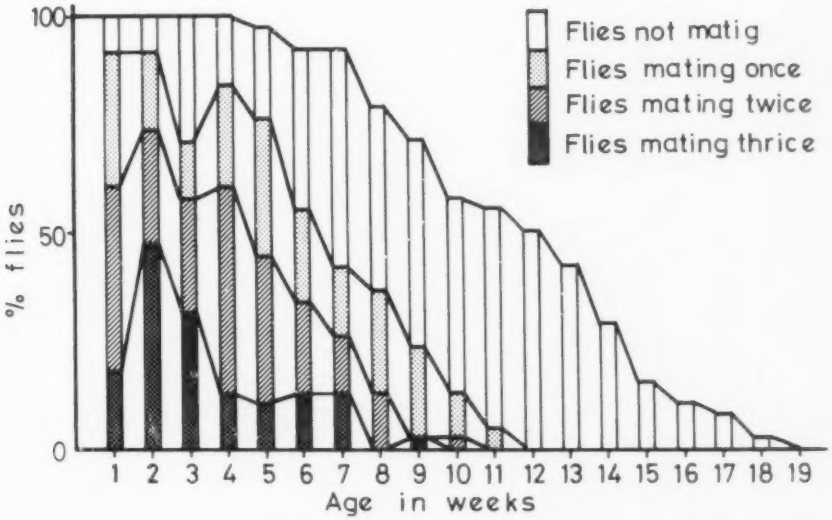

FIG. 1. Age-associated changes in the mating activity of males. 38 flies were kept individually. Activity tests were conducted from 1 to 13 weeks.

TABLE 1. MATING ACTIVITY OF MALE *DROSOPHILA VIRILIS*

Age in weeks	*No. of survivors*	*No. of copulations*	
		Mean ± S.E.	*C.V. (%)*
1	38	1.74 ± 0.14	49.52
2	38	2.13 ± 0.16	46.49
3	38	1.60 ± 0.20	76.00
4	38	1.63 ± 0.15	55.96
5	37	1.35 ± 0.16	70.24
6	35	1.11 ± 0.19	99.20
7	35	0.89 ± 0.19	127.77
8	33	0.58 ± 0.13	130.48
9	27	0.41 ± 0.13	170.32
10	23	0.26 ± 0.11	207.32
11	21	0.10 ± 0.07	315.83
12	20	0 ± –	–
13	16	0 ± –	–

weeks after eclosion during which no males died. The mean number of copulations per male per hour exceeded 1.6 throughout the period, revealing a peak (2.13) at two weeks of age. Then it decreased linearly, arriving at 0.1 for 11-weeks old males. At this age more than 90% of surviving males did not copulate at all. At, and after, 12 weeks of age, no male copulated under the test conditions, even though more than half of the males still survived at this stage.

Fertility. The fertility of aging males was measured as the number of progeny produced by ten-days old females which mated with the tested males. Figure 2 represents the age-associated changes in the mean number of eggs and of progeny per copulated female. The proportion of progeny surviving to adulthood is also shown. The number of eggs laid by a mated female during the 24 hours period was less than 50 with one-week old males and with 11-week old males. With two to ten week old males, however, the number seemed to be nearly constant (about 70 eggs per day) irrespective of the age of male flies. In contrast, the number of progeny per copulated female gradually declined with the age of males, with the exception of one week after eclosion at which time *Drosophila virilis* was not sexually mature. This decreasing tendency associated with male age is more apparent in the case of the proportion of progeny surviving to eclosion. The proportion was more than 80% for one-week old males but decreased linearly until a level of about 35% for nine-week old males (regression coefficient $r = 0.928$, $p < 0.001$).

Age-associated changes in female reproductive activity

Egg-laying activity for individually aged females was examined as a measure of female reproductive activity at one-week intervals throughout their lifespan. Copulations were observed for 33 of 41 tested females. About half of them (17/33) mated more than twice during their lifetime. The mean lifetime number of copulations was 2.2 per female with only small individual variations. The number of progeny produced per female was also recorded but the number was highly dependent on the frequency of copulation. These two

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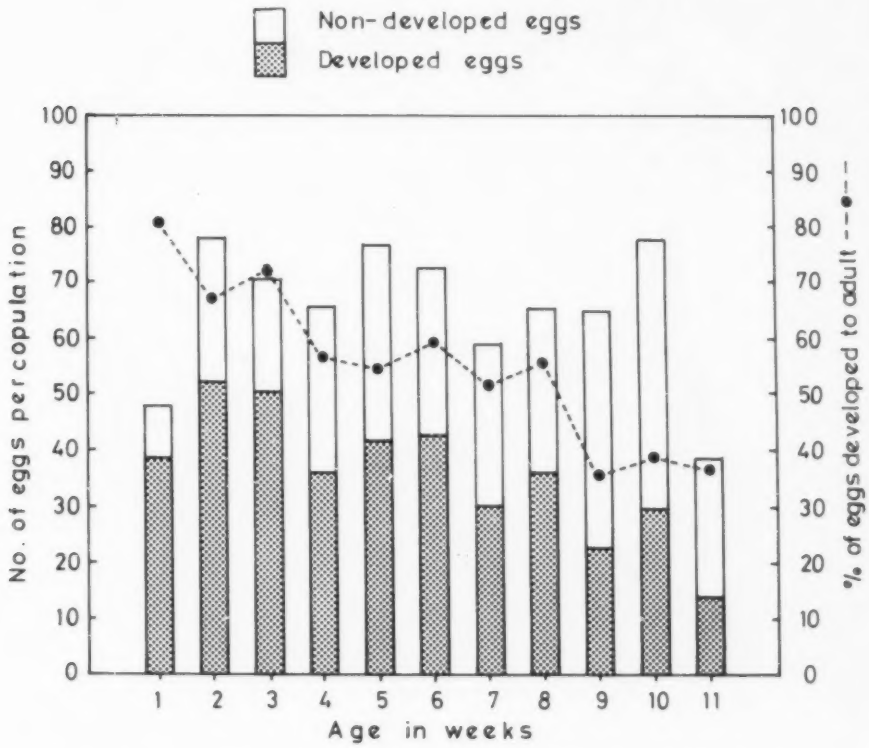

FIG. 2. Effects of the male age on the number and viability of eggs laid by females.

parameters, frequency of copulation and number of progeny, seem to be inadequate as a measure of female reproductive activity. In this experiment egg-laying activity expressed in terms of the mean daily number of eggs deposited by a female was adopted as a more appropriate parameter of female reproductive activity.

Table 2 represents the age-associated changes in the mean number of eggs per female per day at one-week intervals. The number was maintained at a high level (about 50 eggs per female per day) for the first three weeks, being a maximum in two-week old females (55.2). After three weeks it dropped suddenly to about 20 eggs until the sixth week and then decreased rapidly to 0.6 for nine-week old females. At ten-weeks of age, all of the 24 surviving females laid no eggs. The coefficient of variation was constant and small for the first three weeks but gradually increased with age. These results indicate that egg-laying activity is a parameter which changes regularly with female age and is appropriate for quantitative analysis of individual variations in female reproductive activity.

Lifespan and its relationship to reproductive activity

Lifespan data of individually aged flies is summarized in Table 3 and Figure 3. The mean lifespan of males was 81.97 days, being slightly longer than that of females, 77.22

TABLE 2. EGG-LAYING ACTIVITY OF FEMALES

Age in weeks	No. of survivors	No. of eggs/female/day	
		Mean ± S.E.	C.V. (%)
1	41	48.63 ± 2.67	35.14
2	41	55.20 ± 2.67	30.93
3	41	47.07 ± 2.47	33.61
4	41	23.63 ± 2.64	71.63
5	41	15.78 ± 2.62	106.45
6	39	18.21 ± 2.62	89.95
7	36	8.64 ± 1.64	113.93
8	33	2.15 ± 0.64	172.00
9	29	0.62 ± 0.27	233.53
10	24	0 –	–
11	20	0 –	–

days. The maximum lifespan was also longer in males (143 days) than in females (124 days). The variation in lifespan, seen in Table 3 as a coefficient of variation values, is a little larger in males than in females. This tendency agrees with results in our many previous experiments in which TK females and males were kept together in mass cultures.

To analyze the relationships between lifespan and reproductive activity for both sexes, age-associated changes in copulating activity (male) or egg-laying activity (female) were individually examined. For this purpose, the tested flies were divided into three groups according to their lifespan and their reproductive activities were compared. In the case of males, groups I, II and III consisted of flies whose lifespan were less than 56 days (mean − S.D.), between 56–108 days, and more than 108 days (mean + S.D.), respectively (Figure 3). The number of flies in each group was 5, 28 and 5, and mean life spans were 43.2, 81.5 and 123.4 days for the group I, II and III, respectively.

Figure 4 shows the age-associated changes in copulating activity of males for the three groups. The mean number of copulations per male per hour was more than two in group I at the age of one week, while in groups II and III the value was about 1.5. In group I the copulating activity was highest at the age of two weeks. Then the activity dropped suddenly to about 1.0 and this level was maintained until six weeks after eclosion after which no copulation was observed in this group. On the other hand, males of the group III revealed lower copulating activity than males in group I, 1.4 at the age of one week and 0.4 at six

TABLE 3. LIFE SPAN, REPRODUCTIVE AND POST-REPRODUCTIVE PERIODS IN WEEKS

	Male		Female	
	Mean ± S.E.	C.V. (%)	Mean ± S.E.	C.V. (%)
Life span	11.27 ± 0.60	32.93	10.61 ± 0.50	30.24
Reproductive period	7.16 ± 0.35	29.86	6.68 ± 0.26	24.69
Postreproductive period	4.11 ± 0.50	75.51	3.93 ± 0.40	65.52

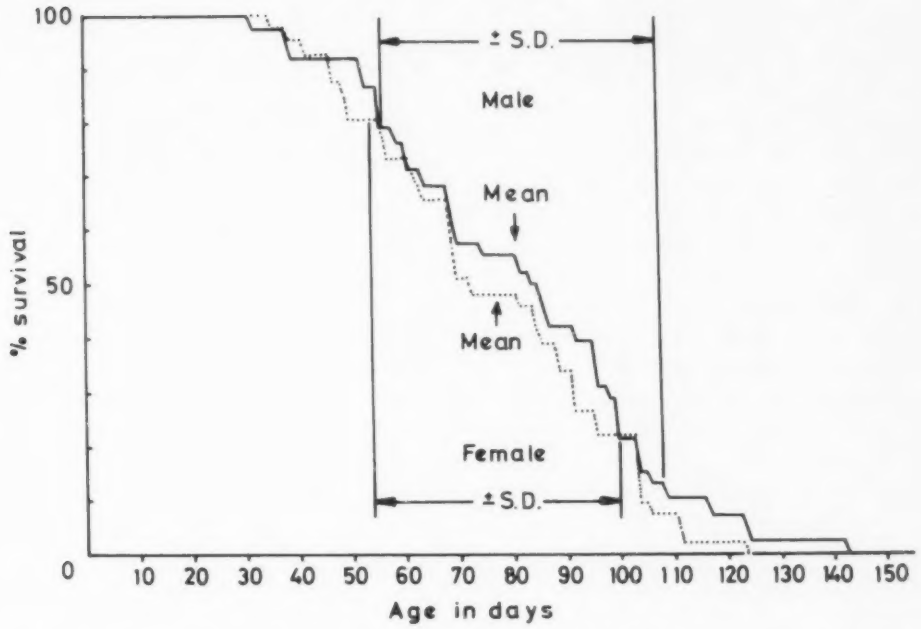

FIG. 3. Survival curves of flies which were kept individually through their lives. Mean lifespans in days were 81.97 and 77.22 for males and females, respectively.

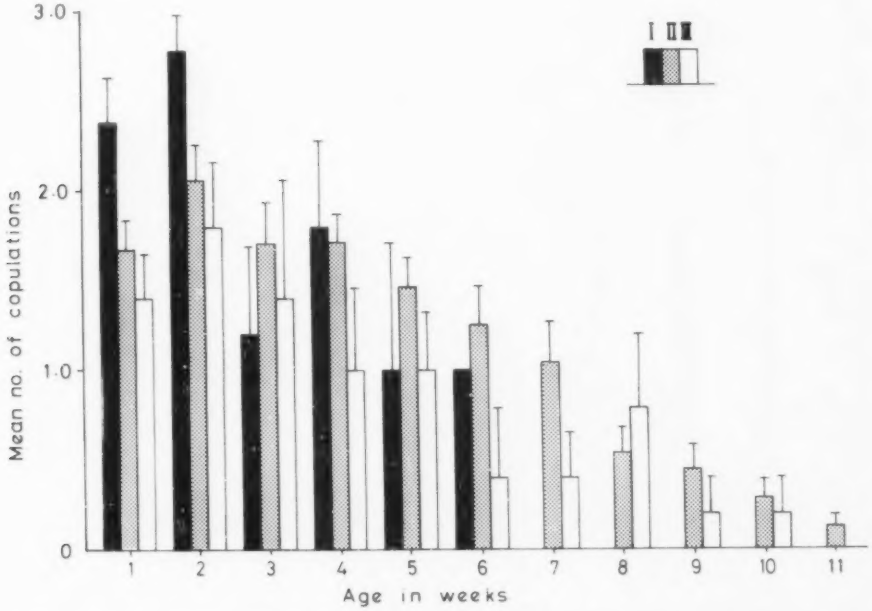

FIG. 4. Comparison of age-associated changes in mating activity of males in three lifespan groups: I, less than 56 days (Mean − S.D.); II, between 56–108 days; III, more than 100 days (Mean + S.D.).

weeks old, but a few males in this group could copulate even at the age of ten weeks. Group II males showed intermediate copulating activity with the last copulations recorded in 11-weeks old males. In all three groups, the peak in copulating activity was found at the age of two-weeks.

For females the data on individual flies were separated into three groups classified by the length of lifespan; that is, group I (flies whose lifespan was less than 54 days), group II (flies with lifespan between 54 and 100 days), and group III (flies lived longer than 100 days). The number of flies in each group was 8, 24 and 9, and mean life spans were 45.0, 76.4 and 108.1 days, respectively. The summarized results are given in Figure 5. During the first three weeks after eclosion, there was little difference in egg-laying activity among these three groups. All females laid about 50 eggs per female per day during this period. This result is quite different from that of male copulating activity described above. After four weeks of age, egg-laying activity of group I females decreased rapidly and no eggs were deposited at and after seven weeks of age. In the groups II and III the decrease was more gradual and the last eggs were laid by nine-week old females in both groups.

Length of reproductive and post-reproductive periods

In this work the reproductive period was defined as the length of life in weeks until the last mating (male) or egg-laying (female) was recorded and the post-reproductive period is defined as the rest of the lifespan. On the average, the reproductive period occupied about

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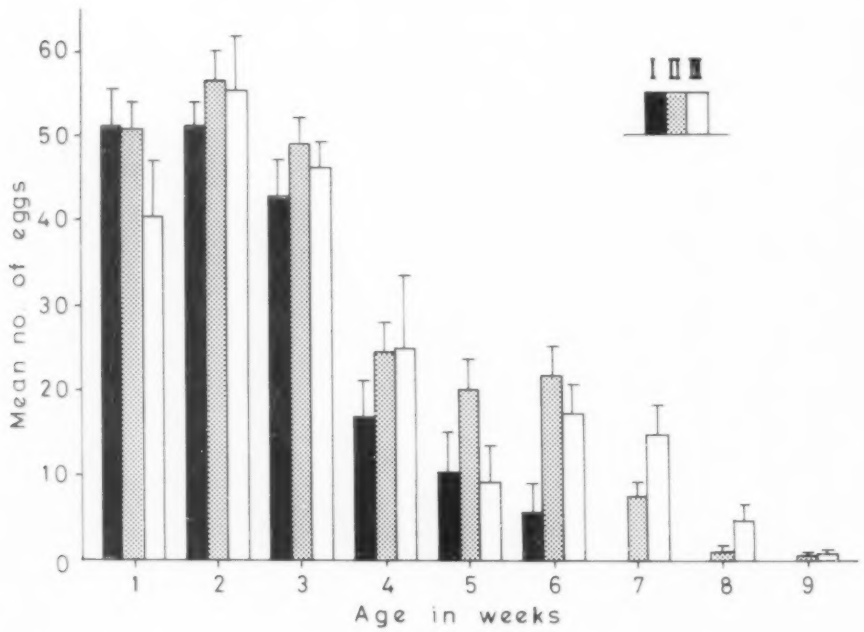

FIG. 5. Comparison of the age-associated changes in the egg-laying activity of females in three lifespan groups: I, less than 54 days (Mean − S.D.); II, between 54–100 days; III, more than 100 days (Mean + S.D.).

60% of the total lifespan in both sexes, 7.16/11.27 weeks in males and 6.68/10.61 weeks in females (Table 3). For individual flies, however, considerable variations were found in the length of both reproductive and post-reproductive periods. Figure 6 represents the relationships between both periods for male flies, classified according to their total lifespan. In male flies whose lifespans were less than ten weeks, the reproductive period lengthened as the total lifespan increased ($r = 0.729$, $p < 0.01$), while no significant changes occurred in the length of the post-reproductive period ($r = 0.349$, $p > 0.05$). In males with lifespans greater than ten weeks, the reproductive period was almost constant (about seven to eight weeks) and the regression coefficient did not significantly differ from 0 ($r = 0.157$, $p > 0.05$). In these males the post-reproductive period increased significantly along with the total lifespan ($r = 0.698$, $p < 0.01$).

Similar tendencies were also observed in female flies (Figure 7). Among females which lived less than ten weeks, the reproductive period increased as flies lived longer ($r = 0.702$, $p < 0.01$) but the post-reproductive period was always around two weeks ($r = 0.350$, $p > 0.05$). For females with lifespans greater than ten weeks, however, a significant increase in the post-reproductive period was apparent ($r = 0.842$, $p < 0.001$) but the reproductive period remained almost constant (seven to eight weeks), giving a regression coefficient of $r = -0.046$ ($p > 0.05$).

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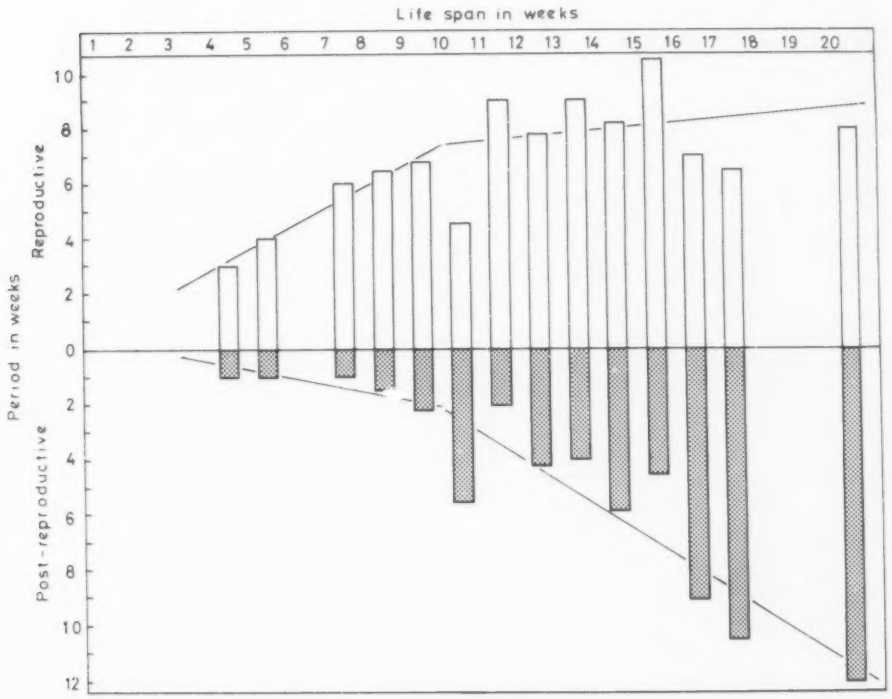
Life span in weeks
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Period in weeks
Reproductive
Post-reproductive
10 8 6 4 2 0 2 4 6 8 10 12

FIG. 6. Relationships between lifespan and reproductive or post reproductive periods in males. Ordinate: mean length of reproductive periods (above) and post reproductive periods (below). Abscissa: lifespan.

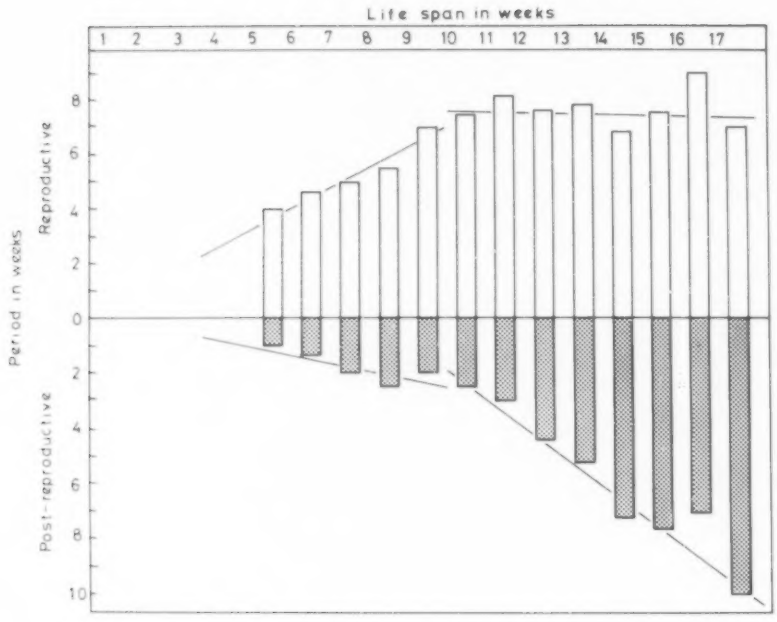

FIG. 7. Relationships between lifespan and reproductive periods in females. Meaning of ordinate and abscissa is same as in Fig. 6.

DISCUSSION

Under standard culture conditions (standard yeast medium III, 25°C, ten females and ten males per vial) females of the TK strain of *D. virilis* always lived longer than males (Aigaki and Ohba, 1983). In the present study flies were individually aged and the mean lifespan of males (81.97 days) was not statistically different from that of females (77.22 days). This agreed with the results of other experiments (Aigaki and Ohba, 1983) in which male mean longevity increased with decreasing male density in culture vials finally approaching the mean longevity of females.

In both sexes reproductive activity reached its maximum about two weeks after eclosion, irrespective of the total lifespan of each fly. In males copulating activity (mean number of copulations per hour) exceeded two at that age, but shorter-lived flies seemed to have a higher mean value and a more rapid decrease after the peak than longer-lived males. In female flies egg-laying activity (number of eggs per day) was relatively constant (50 or more) at one to three weeks of age.

As a result of careful analysis of the length of the reproductive period (the length of life until the time at which last copulation or last oviposition was recorded) and the post-reproductive period of individual flies, the following relationships between the total lifespan and reproductive period can be stated. On the average, the reproductive period occupied about 60% of the total lifespan. However, the proportion of the reproductive and post-reproductive periods varied regularly depending on the total lifespan. In flies whose lifespan was shorter than ten weeks, the reproductive period increased with the total lifespan while the post-reproductive period remained almost constant (less than two

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weeks). In flies, whose lifespan was longer than ten weeks, the post-reproductive period increased while the reproductive period was nearly constant, eight weeks. These phenomena suggest the possibility that the termination of reproductive activity can be used as an indicator of the expected lifespan for individual flies. If the reproductive activity (copulation in males or oviposition in females) of a fly ends before the age of about ten weeks, the death of the fly can be expected within one to two weeks. For flies whose reproductive activity continue to the age of ten weeks, the remaining lifespan can be expected to vary widely from two to ten weeks.

Reproductive activity and lifespan in *D. virilis* show a nearly normal distribution in the genetically homogeneous population. However, reproductive activity does not correlate simply with lifespan in individual flies. Therefore death due to aging and age-associated changes in reproductive activity can be considered as under the control of different physiological processes.

SUMMARY

In the TK strain of *Drosophila virilis,* age-associated changes in reproductive activities (copulating activity and fertility in males and egg-laying activity in females) and lifespan were examined in individual flies. The mean lifespan of individually aged flies was about 11 weeks for both males and females. Copulating activity was maximum in two-week old males, then decreased gradually and finally ended at the age of 13 weeks at which about half of males were still surviving. Females also had a peak in egg-laying activity at the age of two weeks while no eggs were laid by females older than ten weeks, although more than half of females were alive at the stage. Analyses of the relationship between reproductive activity and lifespan of individual flies revealed that shorter lived males exhibited a higher copulating activity in the early stage of their lives than longer lived males. In males whose lifespan was less than ten weeks the reproductive period increased with the lifespan while the post-reproductive period was almost constant (one to two weeks). In males living longer than ten weeks, the reproductive period remained constant (about eight weeks) while the post-reproductive period increased in parallel with the total lifespan. Similar tendencies were also observed in the egg-laying activity of female flies.

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BODY TEMPERATURE OF C57BL/6J MICE WITH AGE

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Abstract—On the basis of a study of rectal temperature in a group of 180, C57BL/6J male mice, ranging in age from 3 months to 30 months, the following conclusions were drawn: 1) The positive correlation between body weight and body temperature typical for rodents was found only for young adults of the C57BL/6J strain; 2) body temperature of male C57BL/6J did not appear to decline until about 23.5 months, after which there was a significant negative correlation ($r = -0.53$) between age and temperature.

INTRODUCTION

AN AGE related decline in basal heat production in humans was described in 1955 by Shock and Yiengst and has since become a well-established observation (Roe, 1970). Surprisingly, body temperature was not included in the list of physiological and behavioral parameters of aging in animals models (biomarkers) as reviewed by the Conference on Nonlethal Biological Markers of Physiological Aging (Schneider *et al.*, 1982). The reason for excluding this parameter may have been the inconsistencies in the literature describing age-related differences in a widely used animal model in gerontological research—the mouse. For example, McLaren (1961) found no evidence of an age-related decline in body temperature across the life span of either sex C57BL/How strain, but there was a significant age-related decrement in the C3H/Bi for both sexes. Subsequently, Eleftherion (1975) reported that body temperature declined with advancing age in the male DBA/2J and C57BL/6J mouse strains. These findings contrasted with those of Leto *et al.* (1976) who did not find any systematic changes with age in rectal temperature of female C57BL/6J mice.

The present study was conducted to clarify this issue and determine whether body temperature may be used as a reliable index of physiological aging.

METHODS AND PROCEDURE

Subjects

Nine age groups, each of 20 male C57BL/6J mice, housed in the colony of the Gerontology Research Center were studied. These animals were housed five–six per cage in plastic cages with wood shavings for bedding. The colony rooms were maintained on a 12 hour light; 12 hour dark cycle, and at a room temperature of 22.5°C ± 0.5. NIH-07 formula food (24% protein) and water were available *ad libitum*, the latter through an automated and filtered system. Only animals that appeared healthy were selected for observation. The mean lifespan of male C57BL/6J mice has been estimated to be 26–27 months in this laboratory (Goodrick, 1975).

Procedure

After measuring the body weight, each animal was placed in specially designed restricter to measure rectal temperature. A Yellow Spring Instrument telethermometer with a series 500 probe was used. The probe was lubricated using petroleum jelly prior to use and was inserted between 2.0 and 2.5 cm into the rectum and held in position for 10 seconds before temperature was determined. Measurements were made once for each animal and were conducted during a 2-hour period 4 hours before light offset.

RESULTS

The mean body temperature and mean body weight for each group is presented in Table 1. An analysis of the relationship between body weight and body temperature within each age group was also conducted. Positive correlations between temperature and body weight existed up to 20 months of age but were significant only at 6, 9, and 12 months ($p < .05$). At 24 months a significant negative correlation appeared, but there were no significant negative correlations for older animals.

In consideration of reports suggesting an age-related decline in body temperature in mice (Eleftherion, 1975), the simple linear regression of body temperature on age was conducted (Figure 1A). The resulting linear regression could be described as follows: $\mathbf{y = 38.1 + (-0.0403 * X)}$, where y is body temperature and X is age (in months). The Pearson correlation coefficient for this relationship was $\mathbf{r = -0.42}$ ($\mathbf{p < 0.001}$). Visual inspection of the raw data suggested that the main contribution to the decline of body temperature with age resulted from a sharp drop in temperature during the last several months of life. To support this observation, further analyses of these data were conducted to determine the possibility of characterising the age related decline in body temperature by two straight lines. By assuming that a minimum three points (20 measures each) will

TABLE 1. MEAN BODY TEMPERATURE AND BODY WEIGHT (± STANDARD DEVIATION) AND CORRELATION (*r*) BETWEEN BODY WEIGHT AND BODY TEMPERATURE FOR DIFFERENT AGE GROUPS OF MALE C57BL/6J MICE.

Age	*Body temperature*		*Body weight*			
(months)	*°C*	*SD*	*gram*	*SD*	*r*	*p*
3	37.50	0.69	26.30	2.47	0.27	n/s
6	38.00	0.52	28.90	4.28	0.55	<0.05
9	37.59	0.74	32.52	2.89	0.45	<0.05
12	37.96	0.79	35.60	5.24	0.56	<0.05
15	37.40	0.81	38.60	3.40	0.34	n/s
20	37.73	0.35	37.70	5.20	0.28	n/s
24	37.54	0.65	38.15	5.70	−0.45	<0.05
28	36.90	0.54	36.25	5.02	0.06	n/s
30	36.28	1.14	33.30	3.50	0.22	n/s

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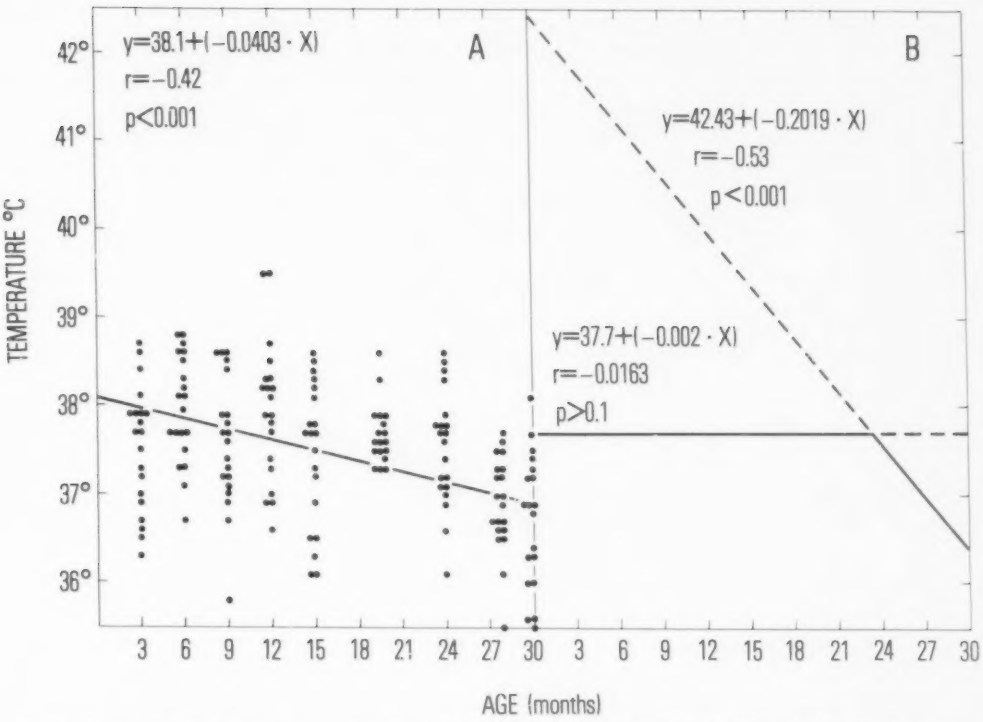

FIG. 1. Changing of the body temperature during the lifespan of the male C57BL/6J mice. **A.** Change in the body temperature during the lifespan. The line through the points is the single line of best fit. **B.** Change in body temperature during the life span represented by two straight lines. The breakpoint occurs at 23.5 months.

determine any straight line, the method of least squares was used to estimate parameters of different straight lines such as 3, 6, 9 months, 3, 6, 9, 12 months, 3, 6, 9, 12, 15 months, and 3, 6, 9, 12, 15, 20 months. The same procedure was applied in the opposite direction—from old to young. The next step was the calculation of the pooled mean square error term (MSE pooled) for the four pairs of lines in order to find out the pair producing the best fit. The result of these calculations are presented in Table 2.

The minimal MSE pooled (0.98743) was produced by the last pair of lines ($p < 0.001$), which suggests that two linear regressions, one between 3 and 20 months and the other

TABLE 2. POOLED MEAN SQUARE ERROR FOR DIFFERENT PAIRS OF LINEAR FUNCTIONS.

First line (months)	*Second line (months)*	*MSE pooled*
3–9	12–30	1.0322
3–12	15–30	1.0286
3–15	20–30	0.99907
3–20	24–30	0.98743

between 24 and 30 months, represent the best fit of age to the temperature data. This graph is presented in Figure 1B. The first linear function (3–20 months) is described by equation: $y = 37.7 + (-0.002 * X)$ – the regression coefficient is not significantly different from zero. The second linear function (24–30 months) is described by the equation $y = 42.43 + (-0.2019 * X)$. The Pearson correlation coefficient representing the relationship between body temperature and age is (-0.53; $p < 0.001$). This analysis indicates the existence of a breakpoint – an age at which a prominent decline in body temperature begins in this strain. This point was calculated using simple equation:

$X = \frac{A_2 - A_1}{b_1 - b_2}$, where A_1 and A_2 are the Y-intercepts of the first and second linear regressions, and b_1 and b_2 are the slopes of these regressions. It was found that this point equals 23.5 months of age.

DISCUSSION

Within the order *Rodentia* a positive correlation exists between species-typical body weights and body temperatures. Within species, this positive correlation is also apparent (Hart, 1971). However, these relationships probably only apply to relatively young animals. Our results with C57BL/6J mice show that a significant positive correlation between body weight and body temperature exists only for mice aged 6–12 months. Moreover, at age 24 months, there is a significant negative correlation. Thus, these data suggest that positive correlations between body weight and body temperature exist only for adult animals before the body weight of individuals is increased by changes resulting from edema or tumors which do not affect body temperature. The change from a positive to a negative relationship between body weight and body temperature at 24 months is of interest with respect to the finding that this age is the inflection point of temperature decline. Our findings indicate that on the average there is no reliable declination in body temperature of C57BL/6J mice up to 23.5 months, but after this there is a sharp drop in body temperature with an estimated loss of 0.2°C for every month of life. The considerable variability of body temperature among all age groups indicates the difficulty of using body temperature as a biomarker of age in small groups of mice. However, the highly significant decline in body temperature after 23.5 months suggests that this parameter may be a useful measurement reflecting an age-related deterioration of a vital homeostatic function since it precedes by about 3 months the age that Goodrick (1975) estimated to be the mean lifespan in the species.

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A TWO-STEP HYPOTHESIS ON THE MECHANISMS OF *IN VITRO* CELL AGING: CELL DIFFERENTIATION FOLLOWED BY INTRINSIC MITOCHONDRIAL MUTAGENESIS

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Abstract—Despite vigorous research, there is yet no agreement on the biochemical mechanisms responsible for the loss of replicative potential of diploid cultured cells. In contrast to the program theories of *in vitro* cell aging, we propose that, as already suggested by Minot in 1907, senescence is the result of cell differentiation. We further maintain that the fundamental cause of cell aging is an instability of the mitochondrial genome because of a lack of balance between mitochondrial repair and the disorganizing effects of oxygen radicals which arise in the respiring mitochondria of terminally differentiated cells. This probably results in intrinsic mitochondrial mutagenesis which may be followed by endonuclease degradation of the altered mitochondrial DNA.

Since the mitochondrial genome controls the synthesis of several hydrophobic proteins of the inner mitochondrial membrane, the postulated denaturation or loss of mtDNA will prevent the replication of the organelles. Thus, deprived of the ability to regenerate their mitochondrial populations, the cells will sustain an irreversible decline in their ability to synthesize ATP, with concomitant senescent degradation of physiological performance and eventual death.

INTRODUCTION

NO GERONTOLOGICAL finding has stimulated more vigorous research than the limited division potential of human WI-38 cells maintained in tissue culture (Hayflick, 1980). However, the specific mechanisms responsible for *in vitro* cell aging remain as elusive as when the phenomenon was first discussed (Hayflick, 1965).

According to earlier hypotheses, the Hayflick limit or phase III phenomenon is caused by accumulation of errors in protein synthesis or by exhaustion of mitotic potential (see reviews on mechanisms of cell aging by Comfort, 1974; Strehler, 1977; Medvedev, 1980). More recently, it has been proposed that the loss of proliferative ability may be the result of cell differentiation rather than of aging (Martin *et al.*, 1973; Kontermann and Bayreuther, 1979; Marek, 1982). As summarized by Martin *et al.*: "such cultures undergo a sort of terminal differentiation *in vitro* analogous to the kinds of terminal differentiation one observes with many types of stem cells *in vivo*, such as hematopoietic cells or myoblasts." Thus, cells stop replicating because "they differentiate themselves to death."

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According to Hayflick (1980), the important fact is that normal cells cultured *in vitro* incur physiological decrements and die. In his words: "Whether one regards this as aging or 'differentiation to death' is more likely to interest semanticists than to lead to meaningful experimentation."

In our view, though it is undeniable that cell differentiation and aging are closely related, they are not identical, since differentiation unfolds under rigid genetic control while senescence can be better explained as the result of metabolic "wear-and-tear." This concept is in agreement with classic views on cell differentiation and aging which will be summarized below as an introduction to our hypothesis that mitochondrial-genetic damage is one of the fundamental mechanisms of cell aging.

CELL DIFFERENTIATION AND AGING

It is well known that some undifferentiated cells such as the protozoan *Amoeba chaos* and the spermatogonia of the mammalian testis do not show aging. On the other hand, highly differentiated cells of the nervous system and muscle show striking alterations with age (Miquel *et al.*, 1977, 1979, 1981). As could be expected, these differences in the way that cells react to the passage of time have inspired numerous analyses, one of which dating back to 1891 is worth remembering:

> Let us now consider how it happened that the multicellular animals and plants, which arose from those unicellular forms of life, came to lose this power of living forever.
>
> The answer to this question is closely bound up with the principle of the division of labour which appeared among multicellular organisms at a very early stage, and which gradually led to the production of greater and greater complexity in their structure.
>
> The first multicellular organism was probably a cluster of similar cells, but these units soon lost their original homogeneity. As the result of mere relative position, some of the cells were especially fitted to provide for the nutrition of the colony, while others undertook the work of reproduction. Hence the single group would come to be divided into two groups of cells, which may be called somatic and reproductive–the cells of the body as opposed to those which are concerned with reproduction.
>
> As the complexity of the Metazoan body increased, the two groups of cells became more sharply separated from each other. Very soon the somatic cells surpassed the reproductive in number, and during this increase they became more and more broken up by the principle of the division of labour into sharply separated systems of tissues. As these changes took place, the power of reproducing large parts of the organism was lost, while the power of reproducing the whole individual became concentrated in the reproductive cells alone.

The above comments by August Weissmann (1891) are in general agreement with the views of another pioneer gerontologist, Charles Minot (1907), who maintained that aging is the price paid for cell differentiation.

As reviewed elsewhere (Miquel *et al.*, 1979), fine structural data from many laboratories, including our own, are in agreement with the above views on the key role of cell differentiation as the trigger of senescence. Thus, in contrast to the lack of subcellular aging changes in actively dividing cells of the tissues of mammalian organisms, irreversibly differentiated cells in nematodes, insects and mammals show clear manifestations of aging. These include a number of cytoplasmic changes, such as lipofuscin increase, ribosomal decrease and changes in mitochondrial number and size. There is also considerable morphological and biochemical evidence suggesting that senescence is accom-

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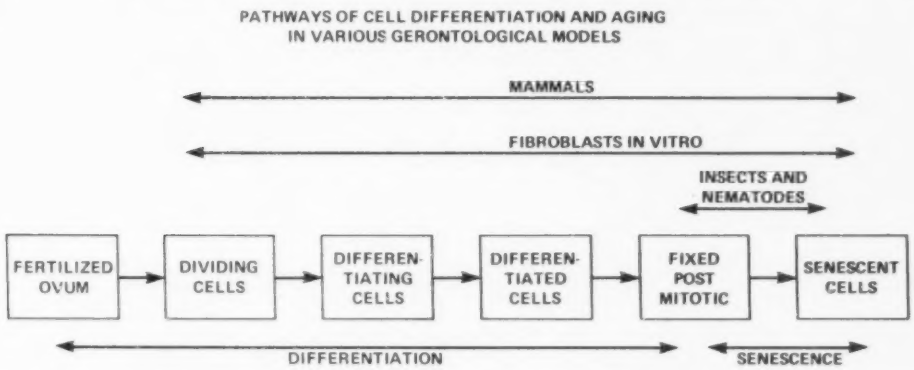

FIG. 1. The study of *in vitro* cell aging is favored by numerous gerontologists on the basis of its supposed cytological simplicity in contrast to the complexity of the *in vivo* histological organization of multicellular animals. However, electron microscopic and cytochemical investigation of aging cells in culture reveals a great variety of cells passing through a differentiation process. Thus, paradoxically, insects and nematodes, which only contain terminally differentiated cells in the mature individuals, may be a more simple model for investigation of the fundamental mechanisms of cell aging than cell cultures.

panied by lipid storage in mitochondria and degradative transformation of these organelles into age pigment (Miquel *et al.*, 1977).

It is of considerable interest towards the development of an unified theory of *in vitro* and *in vivo* cell aging that, as described in detail elsewhere (Johnson, 1979), degenerative changes similar to the above have been seen in old cultures of human fibroblasts. Further, the fact that, after many replications *in vitro,* the fibroblasts show an increase in the number of microvilli suggests that they are undergoing a process of cell differentiation. This concept is also supported by biochemical studies showing that cytochrome oxidase (which is a marker enzyme for the inner mitochondrial membrane) increases its specific activity by 300% in WI-38 fibroblasts aging *in vitro* (Sun *et al.*, 1975). Since it is well known that *in vivo* cell differentiation is accompanied by an increase in respiratory enzymes, that finding is in agreement with the hypothesis that cells undergo a process of differentiation under standard culture conditions Figure 1).

BIOCHEMICAL MECHANISMS OF AGING IN CELLS WHICH HAVE CEASED REPLICATING

Following terminal differentiation, fixed postmitotic cells may start aging because of certain characteristics which are intertwined with the differentiated state (Miquel *et al.*, 1977). These include: *1*) loss of the regenerative effects of mitosis, which, as pointed out by Samis (1966), is an effective filter for elimination of deleterious changes in the informational macromolecules of the genome; and *2*) high levels of oxygen utilization, with concomitant release in the mitochondria of toxic by-products of respiration such as superoxide radical, hydrogen peroxide and lipid peroxides (Harman, 1956; Tappel and Zalkin, 1959; Hornsby, 1982).

The finding that mitochondria contain their own DNA (Fig. 2), which is qualitatively distinct from the nuclear genome, supports the view that mitochondria may sustain intrinsic genetic damage (Economos *et al.*, 1980). This is in agreement with recent studies showing

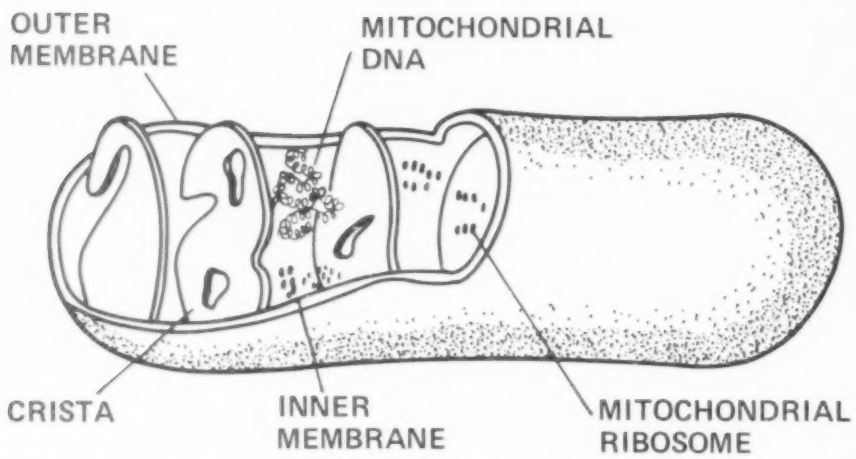

FIG. 2. Diagram showing the location of mitochondrial DNA (mtDNA) in the proximity of the oxygen-radical generating system in the cristae of these organelles (redrawn after Beales and Knowles, 1972). The high levels of hydrogen peroxide and superoxide released in the mitochondria of differentiated cells probably induce damage to mtDNA, which the mitochondria may be unable to counteract because of their lack of scission repair. Since the mitochondrial genome is needed for mitochondrial replication, the postulated mtDNA mutation (probably followed by endonuclease digestion of the altered DNA) will result in an inability to replace the mitochondria lost because of peroxidative attack to the membranes and lysosomal digestion.

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that mtDNA is quite sensitive to attack by toxic chemicals. The formation of complexes between these substances and mtDNA may be favored by the closed circular structure of the mitochondrial genome (Wunderlich *et al.*, 1970, 1972) and probably involves intercalation of the foreign chemical in the DNA. Thus, the mechanism may be similar to that responsible for the mutagenic effects of ethidium bromide and acridine dyes on mtDNA (Bauer and Vinograd, 1968; Slonimski *et al.*, 1968).

Theoretically, the damage caused to mtDNA by mutagenic substances could be repaired by mechanisms similar to those protecting the identity of the nuclear genome. However, recent research suggests that mtDNA may be lacking adequate repair mechanisms (Fukanaga and Yielding, 1979). Specifically, the mitochondrial genome seems to be unable to repair strand breaks. Since it has been shown that hydrogen peroxide (which is normally present in the mitochondria) induces single strand scissions and cross-linking of DNA (Samis *et al.*, 1972), the potential for intrinsic mitochondrial mutagenesis clearly exists (Figure 3).

Despite the apparent vulnerability of mtDNA, which is likely to result in frequent mitochondrial mutation or loss, it is conceivable that an aging cell could maintain a full complement of functionally competent mitochondria. This could be accomplished by *de novo* formation of mitochondria following selective digestion of the organelles which had sustained irreversible damage to their genome. Evidence that mitochondria turn over in mammalian tissues has been obtained using radioactive tracer techniques (Menzies and Gold, 1971). However, this comparative study suggests that the mitochondria turn over much more slowly in the terminally differentiated cells such as the neurons than in the fast replicating cells of the stem cell pools. Further, differentiated cells require high levels of O_2 utilization in order to support their specialized function. Thus, the resulting formation of

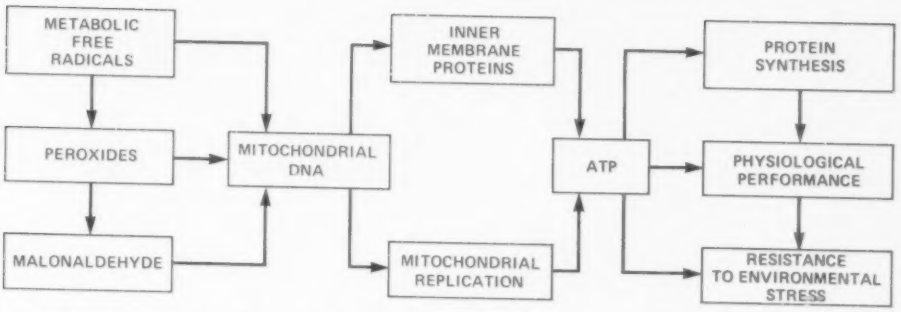

FIG. 3. The postulated damage to the mitochondrial genome provides an explanation for aging at different levels of biological organization. Thus, numerical loss of mitochondria will result in a decline in ATP production, which, in turn, will interfere with protein synthesis and with preservation of the nuclear genome, and (at the physiological level) with performance and survival of the organism. (Reproduced from Fleming *et al.*, 1982).

H_2O_2, lipid peroxides and oxygen radicals may result in mtDNA inactivation or mutation in this cell type. There could even be a total loss of the altered mitochondrial genome because of endonuclease digestion, which would result in an inability to regenerate the mitochondrial population, since mtDNA is required for the synthesis of the hydrophobic proteins of the inner membrane (Tzagoloff *et al.*, 1979).

Since, as noted above, fibroblasts differentiate *in vitro*, the proposed mechanism of mitochondrial-genetic damage provides a reasonable explanation for the senescence of cultures. Thus, both *in vitro* and *in vivo* cell aging would be the result of two different but interrelated processes, that is cell differentiation (which results in a lack of balance between peroxidative disorganization of mitochondria and mitochondrial regeneration) and intrinsic mitochondrial senescence linked to mtDNA mutagenesis (Economos *et al.*, 1980; Miquel *et al.*, 1980; Fleming *et al.*, 1982).

In an evolutionary context, the above is in agreement with the concept that cell aging is the result of selection of genes programming cell differentiation, which, in turn, results in a non-programmed but unavoidable senescent disorganization because of the damaging oxygen radicals and H_2O_2 which are a by-product of the high respiration rate of differentiated cells.

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AGE-RELATED CHANGES IN AMINO ACID POOL SIZES IN THE ADULT SILKMOTH, *BOMBYX MORI*, REARED AT LOW AND HIGH TEMPERATURE; A BIOCHEMICAL EXAMINATION OF THE RATE-OF-LIVING THEORY AND UREA ACCUMULATION WHEN REARED AT HIGH TEMPERATURE

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Abstract—To examine the rate-of-living theory, age-related changes in amino acid pool sizes were investigated in the adult silkmoth, *Bombyx mori*, reared at low and high temperature. At either temperature concentrations of free amino acids contained in silkmoths revealed a great sexual difference. Those in females were generally much higher than in males and the former changed much more dynamically than the latter. Major amino acids or ninhydrin-positive compounds inclusive of some essential amino acids such as Leu, Ile, Val, Thr, Arg, Phe, Met, Ala, Tyr, Gln, Aspn, Lan, Cysta, GABA and PEA accumulated in 4 °C-moths. However, the levels of these amino acids in 35 °C-moths were higher than those in normal 25 °C-moths. Other amino acid levels changed irregularly with advanced age. Inhibition of protein synthesis may occur generally at low temperature, while protein degradation may be promoted at high temperature. Higher concentrations of MSO and Tau in the moths reared at high temperature than in the normal moths suggested also catabolism of amino acids proceeding together with protein degradation at high temperature. Amino acid metabolism seems to be complicated under various temperature conditions.

When reared at the optimal temperature of 25 °C, urea is not present in the body of the silkmoth except for a slight amount in the secreted meconium. In silkmoths reared at the higher temperature of 35 °C, however, an extraordinary accumulation of urea occurs accompanied by a reduction in lifespan by one half. Undoubtedly, urea is produced in this terrestrial insect, although the accumulation mechanism is not clear: in silkmoths reared at various temperatures, arginase is found, but urease is not detected. Arginase activity was found to be higher in male moths than in female moths regardless of the rearing temperature. High temperature rearing also did not induce activity and female activity never exceeded that in males at either 25 °C or 35 °C rearing. Protein degradation accelerated by rearing at high temperatures may result in increased amounts of free arginine, which could cause the active production of urea. This possibility would be a counter-argument to the rate of living theory relating to longevity and temperature. However, at least the above facts signify that an extrinsic factor influences the longevity of an animal by altering its intrinsic aging process.

INTRODUCTION

IT HAS been well-known that exposure either at higher or lower temperature affects the lifespan of poikilothermal animals (Smith, 1957; Hollingthworth, 1966; Parsons, 1977). A similar temperature effect on lifespan was confirmed in adult silkmoths (Osanai, 1979).

When reared at 4 °C, the lifespan was lengthened about five-fold than when reared at 25 °C, the optimal temperature for the insect. However, the minimal level of decreased body weight at death of the moth kept at the lower temperature remained about twice the minimal level of the moth at 25 °C. The moth kept at 4 °C dies before consuming the accumulated substances necessary to maintain its lifetime. This fact suggested distortion and disturbance of metabolisms resulted from rearing at non-optimal temperatures. It is not surprising that temperature treatments cause some abnormalities of metabolism, especially those of nitrogenous compounds.

Since the adult silkmoth, *Bombyx mori,* can not feed after emergence because of the degeneration of its mouth, it can be regarded as a biochemically closed system. This should be emphasized in regard to the metabolism of nitrogen-containing substances. The catabolites formed in the pupal stages as well as in adult are excreted in meconium or stored in the integment. The effects of exposure at varied temperatures on the changes in amino acid pool sizes are presented in this paper.

Quantitative increase in some amino acids such as taurine, α-alanine and β-alanine was found in aged male fruit fly (Chen, 1972), whereas only taurine was accumulated in the silkmoth with advanced age (Osanai and Kikuta, 1981). This paper also examines what amino acid increases occur in aging silkmoths when reared at lower and higher temperatures.

MATERIAL AND METHODS

5-Instar larvae of a strain, Daizo, of silkworm, *Bombyx mori,* were kindly supplied by Dr. T. Gamo, of the Sericultural Experiment Station, Tsukuba. After metamorphosis fresh pupae were divided into two groups according to sex and allowed to develop in the incubator at 25 °C with r.h. of about 60%. Newly emerged adults, not yet mated, were collected every day. Each moth was kept separately in a plastic Petri-dish with a cover (55 mm in diameter and 13 mm high) at 0 °C, 25 °C or 35 °C, as described previously (Osanai, 1979).

One adult silkmoth at each stage was homogenized in 1% picric acid. After deproteinization picric acid was removed by application of a column of Dowex-1. The obtained clear solution was then lyophilized and analysed on an amino acid autoanalyser, JCL-6AH (Japan Electron Optic Laboratory) by developing with both buffer systems composed of lithium and sodium citrate (Osanai and Kikuta, 1981). Arginase activity and urease activity were determined according to methods of Brown and Cohen (1959), and Robinson and Baker (1939), respectively. Protein concentrations were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

RESULTS

1. Lifespans of adult silkmoth reared at different temperatures

The relation between rearing temperature and lifespan of the silkmoth is shown in Figure 1. Since this relation agrees with the Arrhenius equation which expresses the effect of temperature on the rate of chemical or enzymic reactions (Lumy *et al.,* 1951), it seemed apparently to support the "rate of living" theory of longevity of animals (Pearl, 1928).

2. Concentrations of free amino acids contained in the silkmoth, secreted meconium and laid eggs

Although the silkmoth must be regarded as a biochemically closed system, it secretes meconium just after emergence, and in females amino acids are transported into eggs and then "excreted" out of the body in a form of oviposition.

Table 1 demonstrates ninhydrin-positive materials in meconium excreted by silkmoths

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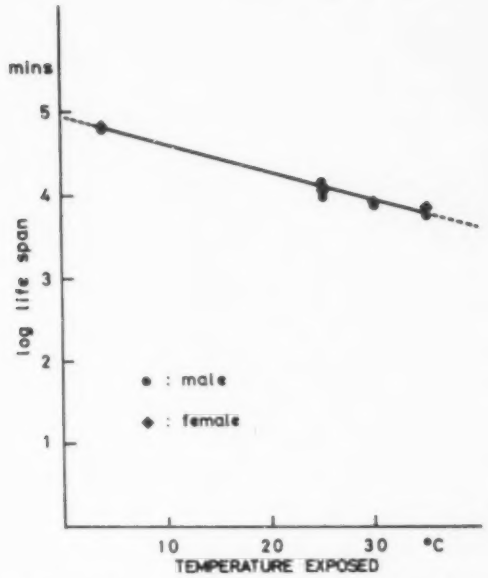

FIG. 1. Relation of the rearing temperature and longevity of the adult silkmoth, *Bombyx mori*.

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reared at 25 °C. Compared with free ninhydrin-positive substances found in the body, the species and quantities contained in meconium are, in absolute terms, very small with most substances barely detectable. Histidine, ammonia and urea were the main constituents of excreta and should be regarded as end products of catabolized nitrogen-containing compounds. Of these three compounds only urea was not detected in the body of the silkmoth reared at 25 °C.

3. Age-related changes in free amino acid levels in the moths reared at low and high temperatures

As described in the previous paper (Osanai and Kikuta, 1981), large sexual differences of the pool changes during imaginal lifetime were observed in most amino acids, especially in lanthionine, cystathionine, methionine sulfoxide, γ-aminobutyric acid.

3-A. Alanine and essential aliphatic amino acids such as leucine, isolucine, valine and threonine. Essential aliphatic amino acids such as Leu, Ileu and Val in the moths reared at optimal temperature fell strongly at the early stage, then kept constant levels or fell a littie in middle age, and finally at the late stage, senescence, the levels changed again, first up and then down (Figure 2). In the 35 °C-moths quantitative reduction of the amino acids proceeded more gradually with advanced age and the level changes at the senescence were not so marked. In the 4 °C-moths the levels fell just after emergence, but these lowered levels were maintained during the whole lifetime. In males a dramatic increase in amino acids was found at senescence, suggesting probable active decomposition of proteins. This demonstrated the similar tendencies at either temperature, but the levels in the moth kept

TABLE 1. FREE NINHYDRIN-POSITIVE SUBSTANCES CONTAINED IN THE WHOLE BODY, MECONIUM AND EGGS OF 4-DAY OLD SILKMOTH (DAIZO-STRAIN) REARED AT 25°C (N MOLES/INSECT)

	Male (n = 3) 0.26 ± 0.04 g		*Female (n = 3) 0.47 ± 0.02 g*		
	Whole body	Meconium	Whole body	Meconium	Eggs
NH_3	909 ± 196	2813 ± 728	4647 ± 1249	893 ± 124	275 ± 112
Urea	–	200 ± 100	–	275 ± 125	–
3M-His	–	–	18 ± 4	–	–
His	1009 ± 308	110 ± 5	2855 ± 669	263 ± 21	270 ± 42
Lys	108 ± 34	–	185 ± 21	–	38 ± 1
Arg	305 ± 38	–	803 ± 80	–	345 ± 15
Ileu	80 ± 14	17 ± 2	190 ± 22	12 ± 1	40 ± 1
Leu	135 ± 20	13 ± 4	258 ± 39	–	52 ± 2
Val	185 ± 35	25 ± 1	403 ± 62	14 ± 2	61 ± 19
Thr	226 ± 60	18 ± 1	527 ± 39	20 ± 3	87 ± 5
Ser	1562 ± 197	12 ± 6	1633 ± 282	13 ± 6	210 ± 14
Gly	351 ± 67	9 ± 2	1268 ± 172	9 ± 4	508 ± 76
Ala	709 ± 78	14 ± 1	1322 ± 177	13 ± 3	463 ± 81
Tyr	199 ± 69	10 ± 1	386 ± 128	–	53 ± 4
Phe	52 ± 36	7 ± 6	35 ± 13	–	–
Asp	6 ± 1	–	24 ± 2	–	–
Aspn	157 ± 65	–	418 ± 51	–	–
Glu	16 ± 6	–	–	–	11 ± 1
Glun	1175 ± 272	–	1622 ± 804	–	–
Pro	228 ± 88	–	254 ± 57	–	–
Met	95 ± 29	–	137 ± 36	6 ± 2	56 ± 7
Lan	384 ± 93	–	2279 ± 414	–	520 ± 37
Cysta	87 ± 19	–	870 ± 40	–	14 ± 2
Tau	1103 ± 102	–	1047 ± 235	–	7 ± 6
MSO	542 ± 144	81 ± 10	143 ± 56	23 ± 10	278 ± 22
Orn	12 ± 3	–	33 ± 20	–	2 ± 1
γ-Ala	73 ± 28	–	190 ± 29	–	–
γABA	36 ± 3	5 ± 3	500 ± 71	11 ± 1	36 ± 2
PEA	134 ± 57	–	568 ± 56	–	731 ± 19
P-Ser	–	–	–	–	10 ± 3

3M-His: 3-methylhistidine, Lan: lanthionine, Cysta: cystathionine, MSO: methionine sufoxide, γABA: γ-aminobuthric acid, PEA: phosphoethanolamine, P-Ser: phosphoserine

at 4°C were maintained throughout the entire lifetime. Age changes in Ala levels were consistent for these essential amino acids at each temperature treatment.

3-B. Easily metabolized amino acids such as glycine, serine and proline. Age-related changes in the levels of Gly and Ser were generally similar to each other. Whereas in the 25°C-moths the levels of amino acids decreased with advanced age, they showed a tendency to increase at lower and higher temperature. Gly in female 4°C-moths reached the maximal level in the middle age, and the amounts were five times as much as at emergence. In male 4°C-moths the maximal levels were not so high, about twice as much at most. By higher temperature exposure the moths in both sexes maintained the levels at emergence or increased them a little.

At the Ser level in 4°C-moths there were hardly quantitative differences between both

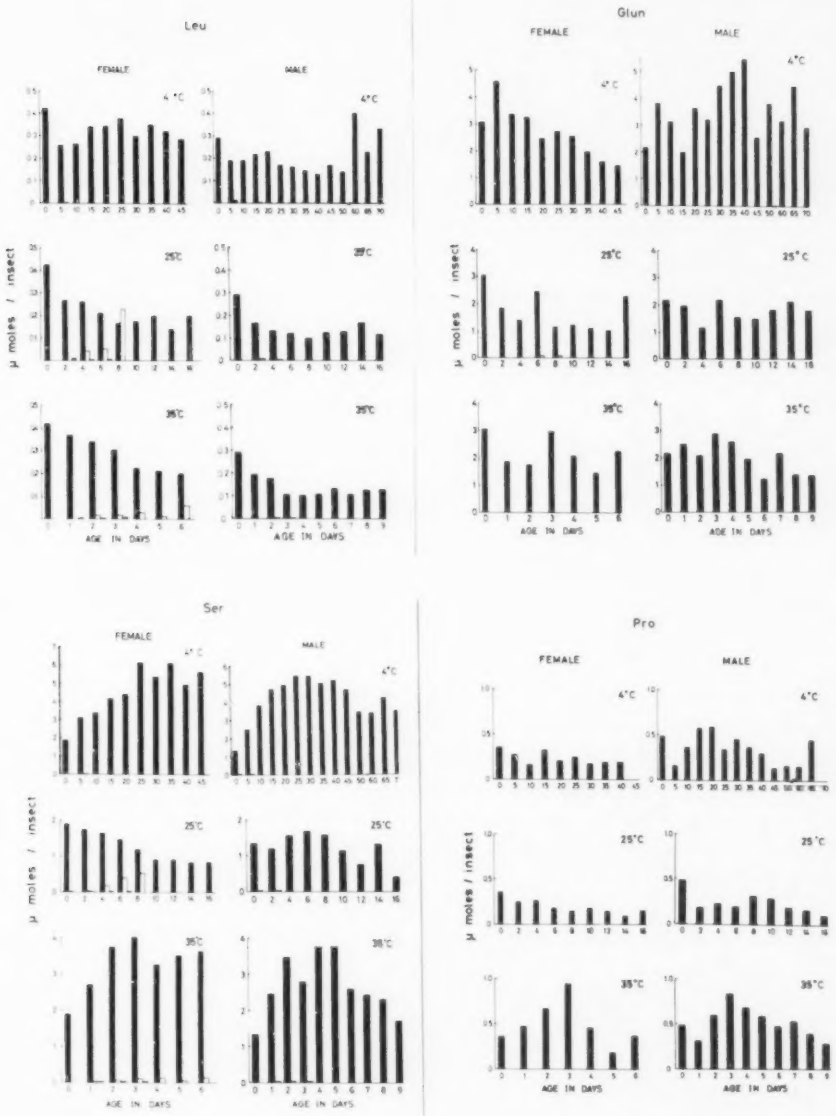

FIG. 2. Age-related changes in contents of leucine (Leu), Glutamine (Glun), serine (Ser) and proline (Pro) in the whole body (filled bars), eggs (open bars) and meconium (half-filled bars) of silkmoths reared at 4 °C, 25 °C and 35 °C. Age scales of 4 °C- and 35 °C-moths (abscissa) are adjusted to correspond with those of 25 °C-moths. Each content (bar height) represents the average value calculated from measurements of five specimens.

sexes, being different from Gly (Figure 2). The levels increased gradually and in middle age reached about three times as much as at emergence. In 35 °C-moths the maximal levels were about twice as much as at emergence. In female moths the level was maintained throughout the entire lifetime, while in males it decreased gradually at senescence.

In 35 °C-moths Pro increased until the middle age and then decreased, whereas its levels

in 25 °C-moths remained almost unvariable (Figure 2). As in female 25 °C-moths Pro kept almost a constant level in female 4 °C-moths, but the 25 °C concentrations were a little higher than the 4 °C concentrations. In male 4 °C-moths an accumulation and following decrease were observed as in male 35 °C-moths. In the males the tendency to show aging level changes in Pro were similar to those in Ser at any temperature, but they are different from each other in females.

3-C. Glutamine and asparagine. Age-related changes in Aspn were scarcely affected by different temperature treatments. The level changes in Glun in 35 °C-moths were not very different from those in 25 °C-moths, but the levels in 4 °C-moths changed dynamically (Figure 2). When kept at low temperature, concentrations of Glun in female moths increased suddenly and then decreased gradually until natural death. The concentrations in males increased gradually until middle age, followed by a gradual decrease.

3-D. Basic amino acids such as arginine, lysine and histidine. Arg and Lys in moths kept at a higher temperature decreased much more gradually than in 25 °C-moths (Figure 3). Both Arg and Lys levels in males showed no change at 4 °C and kept the level at emergence throughout the entire lifetime. Lys in females at 4 °C increased in middle age and the level was stable until natural death. Arg demonstraed a similar tendency, but decreased gradually from middle age.

Aged changes in His tended towards a higher consuming rate in moths reared at the higher temperature (Figure 4).

3-E. Aromatic amino acids such as phenylalanine and tyrosine. Tyr did not show nearly any differences of age level changes in the moths kept at either temperatures, although the moths kept at 4 °C and 35 °C had a tendency to hold Tyr levels, apart from a rapid decrease just after emergence (Figure 3). Phe levels changed irregularly with advanced age under different temperature treatments. In 4 °C-moths higher Phe levels were maintained and this tendency was observed also in 35 °C-moths (Figure 3). Free tryptophan was not detected at all in any cases.

3-F. Sulfur-containing amino acids. Lanthionine and cystathionine revealed a marked quantitative difference between sexes (Figure 5). In male moths the amounts were too little to find differences by any temperature treatments, especially in male 4 °C-moths. Lanthionine decreased with advanced age in female moths at 4°C and 35°C, whereas the moths demonstrated a level decrease: the higher the temperature, the faster the decrease. Lanthionine decreased with advanced age in female moths at 4°C and 35°C, whereas the amounts of this amino acid fell rapidly just after emergence, in moths reared at the optimal temperature, and thereafter enhanced gradually. Met levels were not so strongly variable with advanced age, but in male 4 °C-moths sudden increase was observed at senescence. In females there were almost no level differences among experimental groups, the concentrations in the male moths reared at 4 °C and 35 °C were a little lower than those at 25 °C (Figure 5).

Taurine either accumulated (at 25 °C and 35 °C) or maintained its levels (at 4 °C), as shown in Figure 5. At the higher temperature there was more Tau accumulation. Tau is one of the good parameters for the aging of the silkmoth.

Methionine sulfoxide (MSO), an endproduct of the sulfur-containing amino acids ac-

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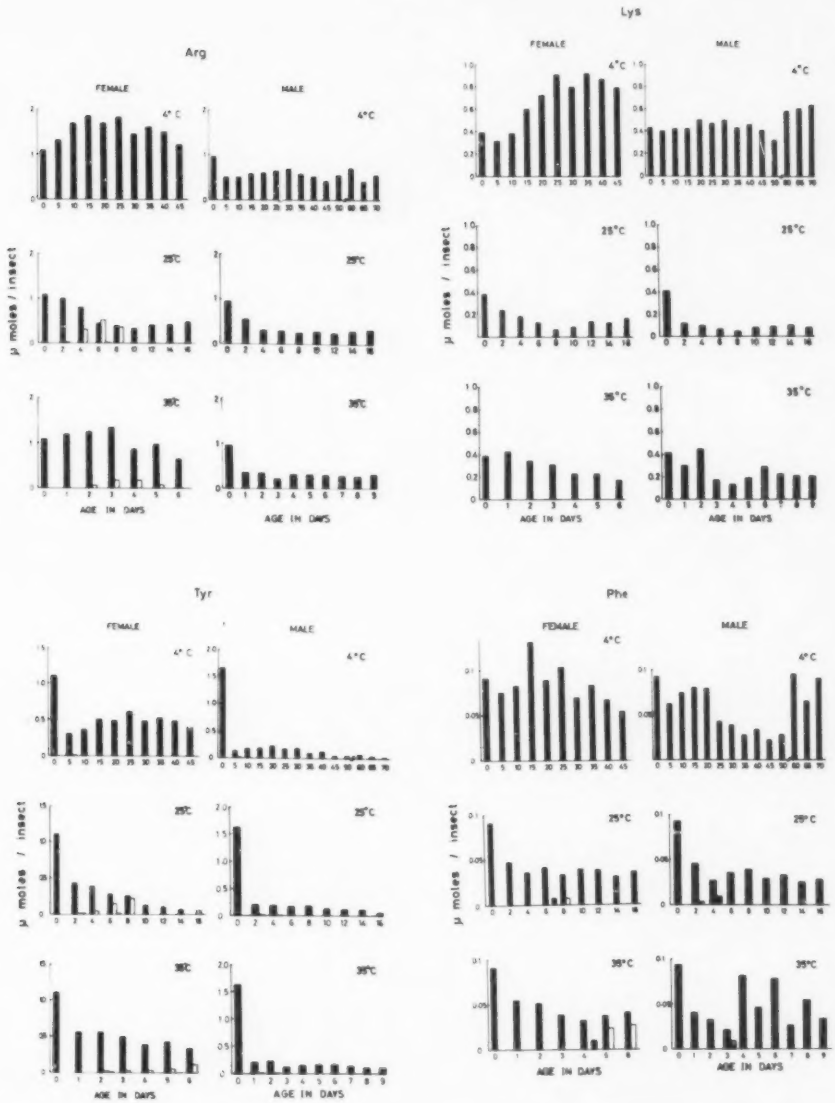

FIG. 3. Age-related changes in contents of arginine (Arg), lysine (Lys), tyrosine (Tyr) and phenylalanine (Phe) in the whole body (filled bars), eggs (open bars) and meconium (half-filled bars) of silkmoths reared at 4 °C, 25 °C and 35 °C. Age scales of 4 °C- and 35 °C-moths (abscissa) are adjusted to correspond with those of 25 °C-moths. Each content (bar height) represents the average value calculated from measurements of five specimens.

cumulated in males much more than in females. The accumulation was most distinct in 4 °C-moths and next in 35 °C-moths (Figure 4). In the moths reared at 25 °C and 35 °C MSO was excreted in meconium and in eggs. Whereas in the moths at 4 °C MSO remained accumulated, in the moths kept at higher temperatures the levels decreased gradually with aging.

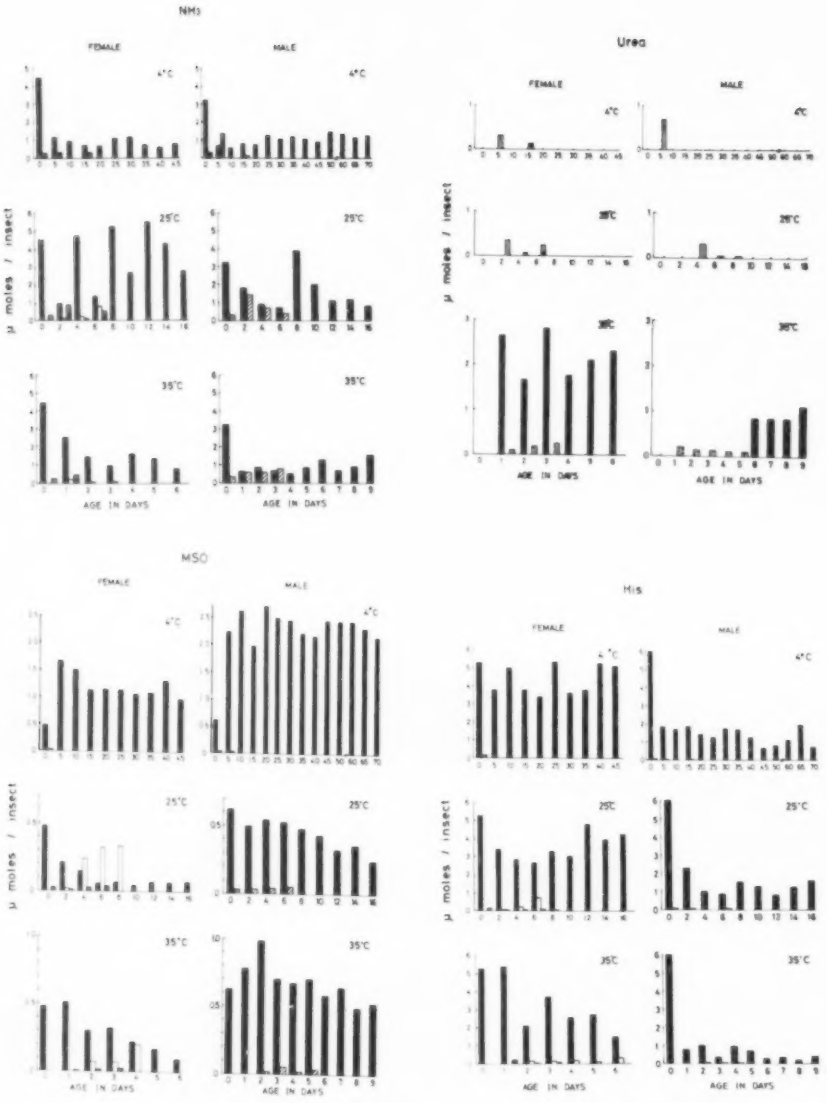

FIG. 4. Age-related changes in contents of ammonia (NH_3), urea, methiosulfoxide (MSO) and histidine (His) in the whole body (filled bars), eggs (open bars) and meconium (half-filled bars) of silkmoths reared at 4 °C, 25 °C and 35 °C. Age scales of 4 °C- and 35°C-moths (abscissa) are adjusted to correspond with those of 25 °C-moths. Each content (bar height) represents the average value calculated from measurements of five specimens.

3-G. End products of nitrogen-compounds such as ammonia and urea. Ammonia levels were very variable in 25 °C-moths (Figure 4). In 35 °C-moths the levels decreased with advanced age, while at 4 °C the levels fell suddenly just after emergence and the lowered levels were maintained throughout the entire lifetime. This substance was secreted in meconium at each temperature.

Considerable accumulation of urea was found in silkmoths reared at 35 °C (Figure 4).

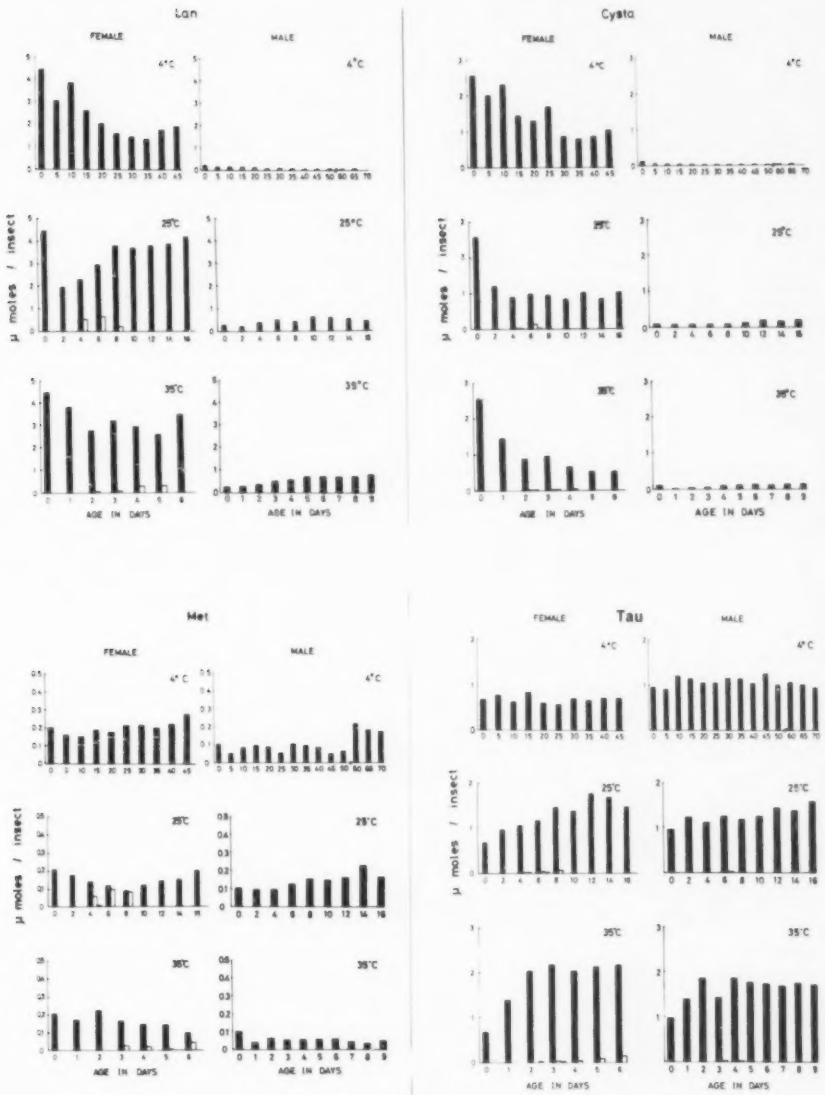

FIG. 5. Age-related changes in contents of sulfur-containing amino acids, lanthionine (Lan), cystathionine (Cysta), methionine (Met) and taurine (Tau) in the whole body (filled bars), eggs (open bars) and meconium (half-filled bars) of silkmoths reared at 4 °C, 25 °C and 35 °C. Age scales of 4 °C- and 35 °C-moths (abscissa) are adjusted to correspond with those of 25 °C-moth. Each content (bar height) represents the average value calculated from measurements of five specimens.

This is the most striking quantitative difference between silkmoths reared at lower temperatures and 35 °C. Male 35 °C-moths excreted more often and more urea than male 25 °C-moths. The accumulation was more marked in females than males. Already after one day of rearing at high temperature, the accumulation begins in females and the quantity reached two or more times that of the males during their whole adult life. In

35 °C-males the accumulation begins on the fifth day after emergence, at the middle stage of adult life. Urea is never found in the eggs laid by female moths reared at any temperatures. Sexual differences in the amino acid pools were already found in silkmoths reared at 25 °C (Osanai and Kikuta, 1981). In the silkmoth reared at higher temperature, protein degradation might be accelerated, resulting in the quantitative increase of free arginine, which causes the active production of urea (Figure 3). While the concentration of free arginine in the male silkmoth hardly varied by higher temperature rearing, the 35 °C-females tended to accumulate the amino acid continuously until about the middle of the adult period. The transport rate of arginine into the ovary was rather slow in moths reared at the high temperature. No excretion of this amino acid was observed in either sex at any temperature. No accumulation of urea was observed in silkmoths reared at 4°C.

3-H. Other ninhydrin-positive compounds. γ-amino butyric acid (GABA) as well as phosphoethanolamine (PEA) revealed quantitatively sexual differences in the silkmoths reared at either temperature. Concentrations of GABA in the males were too low to show aging level changes at any temperatures (Figure 6). In 25 °C-females GABA increased after emergence, reached the maximum in the young age, and then decreased rather steeply. In 35 °C-females the increase was retarded until middle age and its rate was small. In 4 °C-females this ninhydrin-positive compound declined from emergence and kept a low level.

PEA concentrations in males were much less than in females at all temperature treatments. They increased gradually throughout the entire male lifetime. On the con-

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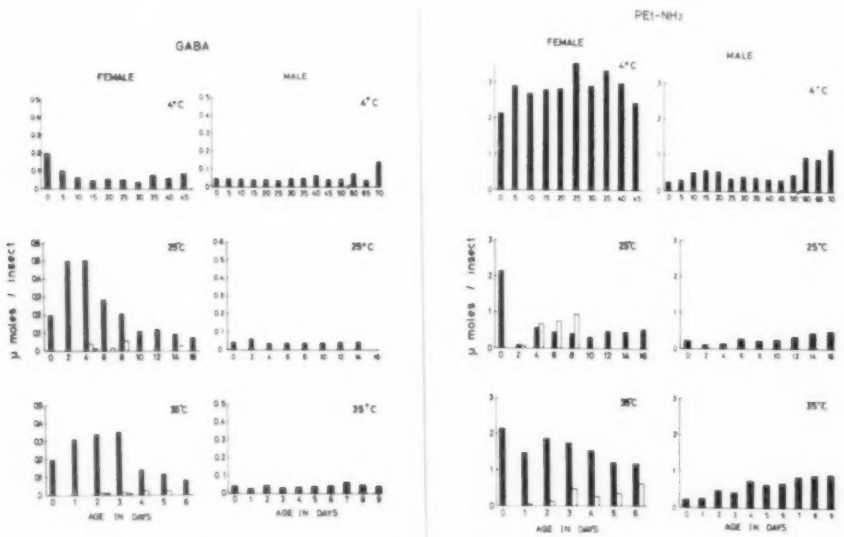

FIG. 6. Age-related changes in contents of γ-amino-butyric acid (GABA) and phosphoethanolamine (PEt-NH_2) in the whole body (filled bars), eggs (open bars) and meconium (half-filled bars) of silkmoths reared at 4 °C, 25 °C and 35 °C. Age scales of 4 °C- and 35 °C-moths (abscissa) are adjusted to correspond with those of 25 °C-moths. Each content (bar height) represents the average value calculated from measurements of five specimens.

trary, in females PEA either fell (at 25 °C and 35 °C) or kept a high level almost constantly during whole lifetime (at 4 °C). In 25 °C-females PEA decreased very rapidly and kept a low niveau until senescence, whereas the decrease rates in 35°C-females were low. This substance was transported into eggs at the 25 °C and 35 °C treatments. PEA should be consumed in part for phospholipid synthesis and in part as energy source. In females this compound must be accumulated by inhibition on metabolic pathways induced by both higher and lower temperature treatments. In 4 °C-females even the transport of PEA into eggs was not observed.

4. Effects of higher temperature rearing on arginase activity

As shown in Figure 4 considerable urea accumulation in moths reared at high temperatures was observed. Urea accumulation was more conspicuous in female than in male moths. These findings can be explained by considering two possibilities: (1) that a high temperature induction of arginase takes place in both sexes; (2) that enzyme activity may be higher in female moths than in males. To verify these possibilities, arginase activities were compared between moths at 35 °C and 25 °C (Figure 7).

There was little difference between activities in males at the two temperatures and little difference for females as well. Female activity was constantly low throughout their whole adult life. 25 °C-male activity reached a maximum at the 2-day-old period and this was five times greater than female activity. Then male activity gradually decreased. In the male moth reared at 35 °C, the maximum was shifted to 1-day-old adults. The results shown in Figure 7 mean that high temperature rearing does not induce arginase activity. However, it might probably explain the accumulation of urea found in moths reared at higher temperatures, that the arginase activity measured *in vitro* must increase as the temperature climbs according to Arrhenius equation. The second possibility, that female

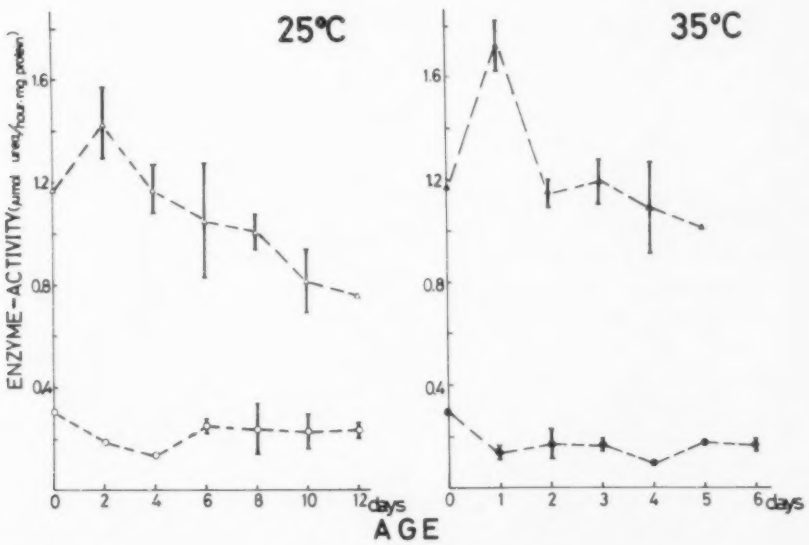

FIG. 7. Effects of higher temperature rearing on arginase activity. Activity is expressed as units per mg protein. Each point represents the mean ± s.d. of five insects. Upper lines: male; lower lines: female.

activity may be higher than in males, is invalidated by the results in Figure 7: male arginase activity is much higher than in females. No urease activity was detected.

DISCUSSION

When aged at higher or lower temperatures, lifespans of poikilothermal animals are generally shortened or lengthened according to the Arrhenius equation in the regions of tolerance for animal life (Hollingsworth, 1969). This suggests that aging is a result of the depletion of some longevity substances, of which consuming rate depends upon temperature (Shaw and Bercaw, 1962).

However, more exact analyses revealed that the short or long lifespan effects of temperature treatments are not so simple. *Drosophila subobscura* could even prolong its lifespan by short exposure to high temperature (Maynard-Smith, 1958), suggesting that this exposure reduces the rate of metabolic processes. Other results that contradict the rate of living theory were brought about by several investigators (Clarke and Maynard-Smith, 1961; Hollingsworth, 1966). From analysis of the death number of the adult silkmoths, Osanai (1978) revealed the presence of some death-sensitive stages during adult lifetime, at which the insect is susceptible to the changes in the environment. An individual having a high sensitivity, which can not bear up against dynamic changes of metabolism occurring discontinuously, dies at earlier stages than the others.

According to the rate of living theory, exposure to various temperatures must cause corresponding changes of metabolic rates. On the contrary, it is also possible that the shortening of longevity when exposed at higher temperature must result from denaturation of proteins as well as structural lipids, followed by occurrence of deleterious changes. We examined these two possibilities in relation to age changes in amino acid pools. Size of an amino acid pool represents a balance between protein synthesis and its degradation, amino acid degradation and its transformation. At the early adult stages the rate of protein synthesis in the silkmoth is much higher than that of other metabolic changes, while at the senescence protein degradation should be accelerated much more than its synthesis (Osanai & Kikuta, 1981). Therefore, in the early adult stages the amino acid level must increase by reduction of metabolic rates at low temperature, causing its accumulation. On the contrary, the level should fall by the promotion of metabolic rates at high temperature.

Accumulations of the essential amino acids such as Leu, Ile, Val and Thr, were observed in 4 °C-moths. This appears to agree with the rate of living theory. However, the levels of such amino acids in 35 °C-moths were higher than those in 25 °C-moths. This may support strongly the hypothesis of protein degradation at high temperature. There were many other amino acids or ninhydrin-positive substances revealing the similar level changes affected by temperature, such as Ala, Arg, Phe, Tyr, Gln, Aspn, Met, Lan, Cysta, GABA and PEA. These findings suggest inhibition on protein synthesis occurs generally at low temperature, whereas protein degradation is promoted at high temperature. Higher concentrations of end products of amino acids, MSO and Tau in the moths reared at high temperature than in the moth at optimum temperature must mean that catabolisms of amino acids are accelerated by high temperature together with protein degradation.

However, amino acid metabolisms seem to be more complicated under various temperature conditions. In spite of 4 °C-rearing Gly, Lys and Ser contained in females increased with age. Moreover, whereas Gly in 35 °C-females held a constant level, Ser in-

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creased with advanced age, and Lys decreased. Aging changes in Ser were similar to each other at 4 °C and 35 °C, but different from those at 25 °C. Pro contained in both sexes, when reared at 35 °C, caused its accumulation until middle age and then fell, and its levels were rather higher than when reared at 4 °C. Aging level changes of GABA in females were similar to those of Pro, but the maximal amounts were found earlier than the stage of Pro, almost at the end of young adult.

Tau, MSO, ammonia and urea, endproducts of nitrogenous compounds, revealed aging level changes without the rules at different temperatures. MSO level changes in females were similar to those of Leu, Val and the others, but the amounts were about half as much as in males. Moreover, MSO in males increased rapidly at 4 °C and reached to a niveau, whereas when kept at 35 °C it revealed a peak at the end of young adults. Tau kept almost a constant niveau at 4 °C, and in the 35 °C-moths it increased rapidly and reached a high level at the early stage and then remained constant. Therefore, Tau must be a good parameter of aging in the silkmoth. Chen (1972) has regarded this ninhydrin-positive substance as one of biochemical parameters for aging *Drosophila*. Ammonia changes very irregularly in 25 °C-moths with advanced age, but in 4 °C- and 35 °C-moths this substance decreased and kept a constant niveau. As shown in Figure 1, effects of temperature on the lifespan of the silkmoth seemed apparently to follow the Arrhenius equation, namely the rate of living theory. However, sometimes metabolic mechanisms fit into the theory, especially at lower temperature, sometimes not, at least at higher temperature.

The most striking quantitative difference between silkmoths reared at 25 °C (and also 4 °C) and 35 °C is the urea accumulated in the body of the short-lived 35 °C-adult of both sexes (Figure 4). This phenomenon was more pronounced in female moths. In terrestrial insects, the main catabolic end product of nitrogenous compounds, such as proteins and nucleic acids, is not ammonia, but uric acid (Gilmour, 1961). Urea is often found as a general constituent of the excretory products of insects, but in small quantities (Wigglesworth, 1950; Powning, 1953). This is attributed to an inactive urea cycle existing in insects, and this fact differentiates insects from terrestrial vertebrates and teleostes (Cochran, 1974).

There have been lively arguments on the urea synthesizing ability of insects (Wigglesworth, 1931; Berridge, 1965). In the blood-sucking bug, *Rhodonius,* excreted urea should originate in its food. However, the self-production of this substance was shown in nymphs of *Dysdercus* from absorption disturbances caused by urea ingested in food. Urea accumulation in the silkmoth reared at 35 °C is *de novo* synthesis in the body of a closed system.

Two metabolic pathways in ureogenesis are so far known: (i) uricolysis in purine decomposing systems via uric acid, allantoin and allantoic acid, (ii) hydrolysis of arginine by arginase in the Krebs-Henseleit urea cycle. The possibility of urea synthesis by the first pathway is not likely in the insect world, because of the metabolic block due to deficiency of allantoicase (Kilby, 1965; Bursell, 1967; Wang and Patton, 1969). However, there is also a little possibility by the second pathway, since the urea cycle does not actively function in insects (Pant and Kumar, 1978). Among urea cycle enzymes only arginase is localized widely in insects (Szarkowska and Porembska, 1959; Kameyama and Miura, 1968; Reddy and Campbell, 1969), and is found also in the silkmoth reared at 35 °C as well as 25 °C (Figure 7). Urea accumulation must be biochemically dependent upon an active balance between arginase and urease. The breakdown of urea to ammonia and carbon dioxide by urease is questionable in insects (Desai and Kilby, 1958). Attempts to find this enzyme

have failed even in *Lucilia* larva which have the ability to produce ammonia (Lennox, 1941). We also were unable to detect urease in the silkmoth reared at various temperatures.

Two possibilities for arginase activity in relation to this urea accumulation can be considered. First, higher temperature might cause induction of the enzyme. Second, the activity in females might be greater than that in males. However, no obvious activity differences were found between moths kept either at high or at normal temperatures as shown in Figure 7. Assuming that silkmoth arginase is stable in the range from 25 °C to 35 °C, it can be concluded that the excessive urea accumulation results simply from a temperature promotion of the enzymatic reaction. The observed sexual differences in arginase activity are completely contradictory to the second possibility. The activity in males is much higher than in females at either temperature. The sex difference in urea accumulation in the silkmoth can not be thus explained by a sex difference in arginase activity. Instead, the phenomenon may be derived from a sexual difference in protein degradation since Pant and Kumar (1978) have reported that in saturniid moths, arginine, the substrate for arginase, is supplied by proteolysis, which might be accelerated by higher temperature.

According to the rate of living theory (Pearl, 1928) the metabolic rate catalysed by enzymatic action increases with a rise in temperature, resulting in a shortening of the lifespan. However, the urea accumulation discovered in the silkmoth reared at higher temperatures suggests that certain metabolic changes in protein and other nitrogen-containing substances occur under this condition, and that the increased rate of enzymatic reactions is not necessarily the only cause of the shortened lifespan. Several phenomena, found mainly in *Drosophila* (Maynard-Smith, 1958; Strehler, 1961), do not fit into the rate of living theory, and these may be caused by similar changes in protein metabolism in advanced ages. Aging may be accelerated for poikilothermal animals reared at higher temperature because of the more rapid accumulation of deleterious or catabolic substances. Urea may be one of these substances.

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ORTHOTOPIC OVARIAN TRANSPLANTATIONS IN YOUNG AND AGED CBA MICE

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Abstract—Reproductive and hormonal changes associated with CBA mice which were orthotopically transplanted with ovaries from young or aged CBA mice were studied. Reproductive decline was defined by the number of mice mating and the number of implantation sites and resorptions in pregnant mice. A high percentage of resorptions was observed in aged mice receiving ovaries from young mice. An increase in the number of resorptions and a decrease in the number of implantation sites was observed in young mice transplanted with aged ovaries. Concentrations of LH, FSH and Prl were analyzed by radioimmunoassay from plasma of pregnant and nonpregnant transplanted mice, ovariectomized and sham-operated controls. Young ovariectomized mice had elevated concentrations of LH and FSH when compared to sham-operated controls. No differences were noted between the aged ovariectomized mice although the gonadotrophin levels in these animals were higher than those in comparable young groups. The highest LH and lowest FSH concentrations were found in young to young transplanted mice. The Prl concentrations in transplanted mice did not vary between groups. These data suggest that the hypothalamic-hypophyseal complex is functioning adequately in all transplanted groups and age-related changes in the ovary and uterus are responsible for embryonic mortality.

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INTRODUCTION

A MAJOR question when examining the aging female reproductive system is whether all organs within the system age at a similar rate or if one or more organs age faster than others, thus limiting the reproductive capacity of the species. One means of establishing whether a particular organ is more susceptible to the aging process is by transplanting tissues from an animal, of a given age, into a host of a different age. Using this approach and employing various transplantation techniques, a number of aging studies have been done on rats (Aschheim, 1964; Sopelak and Butcher, 1982), mice (Jones and Krohn, 1959; 1961a, b) and hamsters (Blaha, 1970). Except for the recent study by Sopelak and Butcher (1982) none of the above studies attempted to measure steroid or gonadotrophin concentrations in animals receiving transplanted tissue.

The CBA strain of mouse is unique because it prematurely loses its ovarian follicles, thus becoming reproductively senescent by 10 to 11 months (Jones and Krohn, 1961a).

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Since a reduction of ovarian follicles in the human may be associated with the onset of menopause, it has been suggested that the CBA mouse may serve as a good experimental model to study age-related changes in the human reproductive system (Gosden *et al.*, 1978).

Using transplantation procedures in the CBA mouse, the following study was conducted by orthotopically grafting ovaries from young or aged mice into young and aged hosts. Plasma and pituitary homogenates from pregnant and nonpregnant mice receiving transplanted ovaries were then analyzed by radioimmunoassay for concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin (Prl). Concentrations of the same three hormones were also assayed from ovariectomized young and aged CBA mice. In addition, a post-implantation mortality frequency was determined in transplanted females by counting the total numbers of mid-gestation implantation sites and the numbers of implantation sites undergoing resorption.

MATERIALS AND METHODS

Animals

Young virgin (6 weeks of age) and retired breeder (7 to 8 mo of age) CBA/CaJ and CBA/T6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). They were housed 3 to 4/cage under uniform temperature (21 to 23°C) and artificial lighting (14 hours light/10 hours dark) and provided with food and water *ad libitum*. Two weeks after receipt of the mice at the University, ovarian transplantations were formed.

Transplantation procedures and mating

The procedure for transplanting ovaries was patterned after that of Stevens *et al.* (1957). Two female mice were simultaneously anaesthetized (donor and recipient, for example young CBA/CaJ and young CBA/T6J, or aged CBA/CaJ and aged CBA/T6J, or young CBA/T6J and aged CBA/CaJ) with 30 mg/Kg ketamine HCl. A dorso-lateral incision was made to expose each ovarian bursa and attached fat pad. A small cut was made in the ovarian bursa (away from the side of the infundibulum) exposing the ovary and allowing for its complete removal by pinching it off at the hilus. Each ovary was either left intact or cut in half with a sterile razor blade and placed in a culture dish containing Medium 199 warmed on a slide warmer (35°C). The ovaries were then exchanged between the two animals by inserting a new ovary into each empty bursa. Once the graft appeared secure within the capsule it was carefully inserted into the body cavity by the fat pad and the skin incision closed by a wound clip. Three weeks after surgery, 2 to 3 transplanted mice were placed with a 2 month-old CBA/T6J male mouse. The T6 substrain was used because its genome is characterized by two small T6 chromosomes which can be detected by chromosomal analysis of the mid-gestation embryos. In this way, the genetic origin of the ova was determined, that is either one or two T6 marker chromosomes would determine the maternal origin of the embryos. If two T6 marker chromosomes were present, then the ovum was derived from the donor ovary (T6) and if only one T6 marker chromosome was visible, then a remnant of the original (recipient, CBA/CaJ) ovary contributed the ovum. Each morning, the female mice were checked for the presence of a vaginal sperm plug. Those mice which mated were killed 10 to 14 days after identifying the plug. Mice which did not mate 2½ months after grafting were examined for 3 to 4 days by vaginal smears and then killed. The females were killed by rapidly bleeding the mice, unanaesthetized, by cardiac puncture. The bleeding is completed in 1 to 2 min and has minimal effects on gonadotrophin and Prl concentrations. At the same time, the procedure is advantageous because it generally results in adequate plasma from individual animals to assay for gonadotrophin and Prl concentrations. The pituitary gland was removed immediately after bleeding and homogenized in 0.5 ml of 0.01 M phosphate buffered saline (pH 7.6), containing 0.05 M EDTA and 0.1% sodium azide. Fifteen young and twenty aged CBA/CaJ mice, which were from the same group and of the same age as mice receiving ovarian transplants, served as controls. These animals, which had not been operated on were checked by vaginal smears and killed at the same time as those mice receiving transplants, but which had failed to mate. Plasma and pituitary homogenates were stored at −85°C. When all samples were collected they were analysed in a single assay, eliminating between assay variability.

Ovariectomy

Young (6 weeks of age) and aged (14 to 15 months of age) CBA/CaJ mice were anaesthetized with Diabutal (sodium pentobarbital). A dorso-lateral incision was made and the ovaries were excised after ligation of the

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ovarian arteries. Mice of the same age were sham-operated as controls. Four weeks following surgery all of the mice were bled unanaesthetized by cardiac puncture. When the sham-operated mice exhibited a prominant diestrous vaginal smear they were killed in the same manner.

Embryo analysis

From each pregnant female, the bicornate uterus was removed, all implantation sites recorded and the number of dead and viable embryos noted. All viable implants were removed individually and prepared for chromosomal analysis. This involved mincing the whole embryos and incubating them in Minimal Essential Media (Grand Island Biological Company) containing colcemid. The cells were then treated in a hypotonic soiution (0.075 M KCl) and fixed in Carnoy's fixative. The metaphase preparations were next placed on slides, air dried and stained in a 2% Giemsa solution. Several metaphases were analysed per embryo to determine the number of T6 marker chromosomes.

Radioimmunoassays

The procedures for assaying mouse LH, FSH and Prl have been described in detailed elsewhere (Parkening *et al.*, 1980). Gonadotrophin concentrations were measured using NIAMDD rat LH and FSH RIA kits. The LH was expressed in terms of the NIAMDD rat LH RP-1 standard and the FSH expressed as ng equivalents of a pooled plasma obtained from CBA mice. Single samples of plasma, 100 μl and 200 μl, were assayed for LH and FSH concentrations. Prl concentrations were measured with an RIA antiserum to mouse Prl (rabbit AFP-1311078) and were expressed in terms of a highly purified mouse Prl (AFP-4111-E) suppled by NIAMDD. Duplicate 50 μl samples were assayed for plasma Prl. Ail samples for a particular hormone were analysed in a single assay and the intra-assay coefficients of variation were 2.2, 2.9 and 4.9% for LH, FSH and Prl, respectively.

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RESULTS

The radioimmunoassay of plasma LH from CBA mice using a NIAMDD rat standard showed good parallelism. The results of the radioimmunoassay of FSH from female CBA mice was not parallel with the rat standard (Figure 1). This was true for FSH from both young and aged mice, whether sham-operated or ovariectomized. A repeat experiment yielded the same results. Serum FSH was expressed, therefore, in terms of a pooled serum obtained from female CBA mice.

Preliminary studies on gonadotrophin secretion indicated that the pituitaries of young ovariectomized CBA mice released at least seven times ($p < 0.001$) more LH and FSH than those of young sham-operated mice (Table 1). In contrast, there were no statistical differences when comparing concentrations of LH and FSH in ovariectomized and sham-operated aged CBA mice. However, gonadotrophin concentrations in both groups of aged mice were considerably higher ($p < 0.001$) than those in young sham-operated animals (Table 1).

The highest percentage of pregnancies achieved after orthotopically transplanting ovaries occurred in the young to young transplantations, where eight to ten mice showing a vaginal sperm plug had implantation sites (Table 2). The least amount of success occurred when transplanting ovaries from aged to aged CBA mice, since only 8 of 55 mice mated and none of the 8 animals contained implantation sites when killed 10 to 14 days after finding the sperm plug. The relatively high number of resorptions (7 to 18) in aged mice having received bilaterally or unilaterally transplanted ovaries from young mice, suggests that a uterine factor is involved in the age-related reproductive decline (Table 2). The quality of ova from aged ovaries transferred into young mice also appears questionable because of the high rate of resorptions (43%) found in the uteri of younger animals. None of the six young mice which became pregnant after receiving a unilateral aged ovarian transplant had any implantation sites in the ipsilateral uterine horn (Table 2).

The mean ovarian weight in mice which received bilateral ovarian transplants was

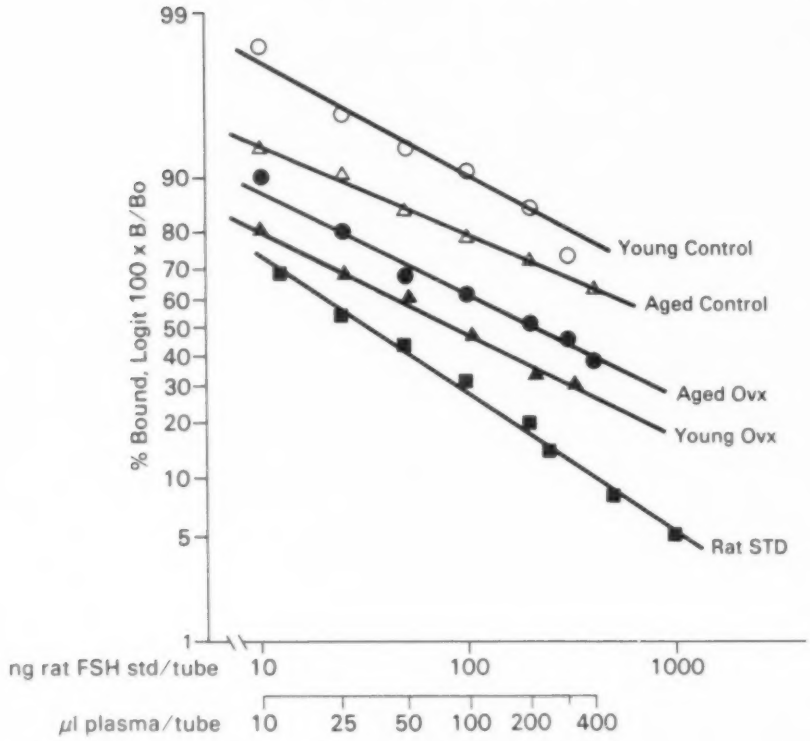

FIG. 1. Dose-response curves for pooled plasma from young and aged control and ovariectomized (Ovx) CBA mice were not parallel with the slope of regression line for the rat FSH RP-1 NIAMDD standard.

greatest in aged mice having received young ovaries (Table 2). This weight, however, was approximately 3 gms lighter than that of ovaries from comparable aged control mice (11.8 ± 0.6, n = 15) which exhibited a diestrous vaginal smear. The same relationship existed when comparing weights of aged diestrous controls (9.3 ± 0.7, n = 15). The mean ovarian weights from transplanted young ovaries into young mice and transplanted aged ovaries into aged mice were lower than that of the other transplanted groups. This dif-

TABLE 1. PLASMA CONCENTRATIONS OF LH AND FSH IN YOUNG AND AGED CONTROL AND OVARIECTOMIZED CBA MICE

Age group of mice	*No.*	*Plasma (Mean ± S.E.M.)* *LH (ng/ml)*	*FSH (ng eq./ml)*
Young (2.5 months old)			
Sham-operated	16	3.3 ± 0.5	125.9 ± 8.8
Ovariectomized	15	24.1 ± 1.8*	1068.4 ± 93.4*
Aged (15.5 months old)			
Sham-operated	22	14.8 ± 1.9	1023.2 ± 122.8*
Ovariectomized	12	18.1 ± 2.2*	794.9 ± 84.8*

*Significantly different from young sham-operated control mice, $P < 0.001$.

TABLE 2. ORTHOTOPICALLY TRANSPLANTED OVARIES IN CBA MICE

No. of mice and type of transplantation	*No. which mated (%)*	*No. pregnant (%)*	*Total implants*		*Total resorptions (%)*		*Mean ± S.E.M.* ovarian wt.		*Plasma (mean ± S.E.M.)* *LH (ng/ml)*	*FSH (ng eq./ml)*	*Prl (ng/ml)*
Bilateral											
15 Young → Young[a]	9 (60)	8 (89)	39		7 (18)		6.2 ± 0.7		12.5 ± 3.6	80.2 ± 28.4	36.7 ± 7.9
10 Young → Aged[b]	4 (40)	2 (50)	10		3 (30)		9.1 ± 0.5		2.0 ± 0.5	199.5 ± 71.5	29.2 ± 5.9
10 Aged → Young[b]	5 (50)	4 (80)	7		3 (43)		6.2 ± 0.7		6.0 ± 3.0	293.7 ± 85.7	40.3 ± 17.8
55 Aged → Aged[a]	8 (15)	0 (0)	0		0		2.5 ± 0.5		–	–	–
			T[c]	*I*[d]	*T*	*I*	*T*	*I*			
Unilateral											
10 Young → Aged[b]	8 (80)	6 (75)	8[f]	17	4 (50)	6 (35)	4.9 ± 1.0	8.7 ± 0.7	1.0 ± 0.2	250.2 ± 62.3	21.9 ± 6.6
10 Aged → Young[b]	10 (100)	6 (60)	0	35	0	0	3.4 ± 0.4[e]	9.1 ± 1.0	4.9 ± 2.1	128.7 ± 9.6	51.0 ± 12.8

[a]Half ovary transplanted
[b]Whole ovary transplanted
[c]T = Transplanted ovary
[d]I = Intact ovary
[e]Two transplanted ovaries were not found and were presumed to have slipped out of ovarian bursae
[f]Only three of the six mice had implantation sites on the side of the transplanted ovary

ference was due to only half an ovary being transferred to the ovarian bursa, rather than a whole ovary. Weights of whole ovaries transferred unilaterally in mice were even lower than those of mice having received whole-ovary bilateral ovarian transplants (Table 2).

Plasma concentrations of LH from young to young transplanted mice were at least twice as high as the concentrations from any of the other transplanted groups (Table 2). Whether this represents a real difference between the groups or is a result of the small sample size of some of the groups is not known. The only statistical differences which existed ($p < 0.05$) were between the young to young and young to aged (unilateral) transplants. The concentrations of LH in aged mice receiving young ovaries tended to be low in both the bilateral and the unilateral transplants.

Plasma concentrations of FSH were lowest in young to young transplanted mice. The mean concentration, however, was significantly different ($p < 0.05$) only from that found in young to aged unilateral transplanted mice (Table 2).

Plasma Prl concentrations, while variable, did not differ statistically between the various transplanted groups (Table 2).

The eight aged mice which received transplanted aged ovaries and which mated were killed 10 to 14 days after detecting vaginal smear plugs. None of these mice appeared to be pregnant as indicated by palpation or by weight gain. However, they were killed to determine if there was any evidence of resorbing implantation sites, of which there were none. All but two of the mice had a vaginal smear consisting predominantly of leukocytes. The hormonal values from these mice, were 15.1 ± 5.4 ng/ml, 655.1 ± 123.1 ng eg./mg and 27.0 ± 8.3 ng/ml for LH, FSH and Prl, respectively.

85% of the 55 aged mice which received ovarian transplants from aged animals did not mate with young fertile male mice. 35 of these mice were subsequently killed ten weeks following the transplantations and their plasma concentrations of LH, FSH and Prl were compared with those of identically-aged control mice (Table 3). The only statistical differences found between transplanted and control mice occurred during diestrus. The concentrations of Prl in transplanted mice which showed a strictly leukocytic vaginal smear were lower ($p < 0.05$) than those of control mice. Other differences in hormonal levels were observed when mice were examined according to the day of the estrous cycle (Table 3). None of the vaginal smears from aged transplanted mice monitored for several days showed the typical cornified cells indicative of estrus.

The remaining 12 aged to aged transplanted mice which did not mate were superovulated (10 I.U. PMSG and 10 I.U. HCG—Collins *et al.*, 1980) to determine if the mice were still capable of ovulating and whether the ova were capable of reaching the oviduct. Corpora lutea were found on the ovaries of five of these mice. The mouse responding the most to exogenous gonadotrophins contained two corpora lutea on the right ovary and three on the left ovary. The oviducts of mice whose ovaries exhibited corpora lutea were flushed for ova. Only two ova were found in the oviduct of one of the mice, suggesting that adhesions or other factors prevented the ova from reaching the Fallopian tubes.

Histological sections of young ovaries transplanted into either young or aged mice contained numerous oocytes in different stages of development and generally showed evidence of corpora lutea. Aged ovaries transplanted into young receipients contained fewer ovarian follicles, but there was consistent evidence of developing follicles and/or corpora lutea. Most of the aged ovaries transplanted into aged mice also contained some

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TABLE 3. CONCENTRATIONS OF PLASMA LH, FSH AND PRL IN AGED CONTROL AND TRANSPLANTED CBA MICE AT DIFFERENT STAGES OF THE ESTROUS CYCLE

Type of vaginal smear	*No. of mice*	*Transplanted mice Plasma (mean ± S.E.M.)*			*No. of mice*	*Control mice Plasma (mean ± S.E.M.)*		
		LH (ng/ml)	*FSH (ng eq./mg)*	*Prl (ng/ml)*		*LH (ng/ml)*	*FSH (ng eq./mg)*	*Prl (ng/ml)*
Only leukocytes	20	30.8 ± 2.6	847.7 ± 49.0	27.5 ± 3.5	7	28.1 ± 4.2	1077.1 ± 146.7	51.4 ± 13.8[e]
Metestrous squamous cells and leukocytes	8	11.1 ± 3.3[c]	517.3 ± 86.4[b]	43.9 ± 9.1[a]	5	18.4 ± 3.8	659.5 ± 42.4	39.6 ± 16.3
Mixed (nucleated cells, leukocytes and cornified cells)	7	7.5 ± 0.9[c]	371.0 ± 90.8[c]	29.9 ± 7.1	5	6.1 ± 1.7[b,d,f]	293.0 ± 90.1[b,e,f]	21.7 ± 7.4
Predominantly cornified cells	0	–	–	–	3	22.6 ± 4.1	834.3 ± 70.1	55.3 ± 19.9

Significantly different from mice within that group having vaginal smears containing only leukocytes: [a]$P < 0.05$, [b]$P < 0.005$, [c]$P < 0.001$.
Significantly different from control mice having metestrous squamous cells and leukocytes: [d]$P < 0.05$, [e]$P < 0.005$.
Significantly different from control mice having predominantly cornified cells: [f]$P < 0.01$.
Significantly different when comparing transplanted and control mice: [g]$P < 0.05$.

large developing follicles, although the numbers of mice whose ovaries contained corpora lutea were fewer than in any other transplantation groups. A few of the ovaries from aged to aged transplantations only contained primary follicles (<30).

DISCUSSION

These data substantiate the use of the CBA mouse as a model to study reproductive aging. In a previous study Gosden *et al.* (1978) suggested that the CBA mouse, because of its premature loss of oocytes, may be a good animal model for studying age-related changes in the human reproductive system. These investigators were somewhat cautious in their statement, because they had found no differences in LH levels between young and aged ovariectomized and sham-operated mice. The data from our study using the same strain, but from a different animal colony, indicate that plasma gonadotrophin and Prl concentrations in these mice do exhibit patterns of change which are similar to those occurring in post-menopausal women. It is concluded therefore, that the female CBA mouse is a good animal model to better understand the effects of aging on the peri- and postmenopausal years.

The finding that plasma FSH dose-response curves from female CBA mice were not parallel with the rat FSH standard curve in the radioimmunoassay was unexpected. Both plasma and pituitary FSH from several other strains of mice have been found to parallel the NIAMDD rat standards (Bronson and Stetson, 1973; Murr *et al.*, 1974; Michael *et al.*, 1980; Parkening *et al.*, 1978, 1980). Whether this is a unique feature of this strain or whether other strains also possess a slightly altered form of FSH awaits further investigation.

The response of the hypothalamic-hypophyseal complex in young and aged ovariectomized CBA mice was similar to that found in young and aged ovariectomized CD-1 and C57BL/6 mice (Parkening *et al.*, 1983). Therefore, it would appear that the elevated concentrations of gonadotrophins in intact, aged CBA mice result from depressed levels of ovarian steroids acting upon the hypothalamic-hypophyseal complex.

The hormonal data suggest that the hypothalamic-pituitary axis is functioning adequately in all transplanted groups, including the aged mice having received aged ovaries. The latter group had plasma LH, FSH and Prl concentrations similar to those from a group of non-operated aged mice. In fact, practically all of the aged mice, whether transplanted or control animals, were still exhibiting a type of estrous cycle. Those mice having predominantly a diestrous vaginal smear contained ovaries bearing the least number of oocytes. However, it is not known why so many of the aged mice which received aged ovarian transplants and exhibited an estrous cycle failed to mate. In these mice half an ovary was transplanted rather than an entire ovary. This was done because higher success rates have been reported using this regimen of ovarian transplantation (Stevens, 1957). Transplanting half an ovary, however, markedly reduces the total number of oocytes, not only because of less ovarian tissue, but also because of an estimated loss of as many as 65% of the oocytes due to ovarian necrosis prior to revascularization (Jones and Krohn, 1960).

Gosden *et al.* (1983) contend that a minimum threshold number of follicles is required to sustain ovulatory activity in the C57BL/6 strain of mouse. If this is true, then the unique nature of premature loss of oocytes in the CBA mouse, combined with the loss of oocytes through grafting procedures, may result in a follicle number which is less than the

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number required to sustain a regular estrous cycle and ovulation. Only 5 of the 12 aged to aged transplants, which were injected with exogenous gonadotrophins, exhibited corpora lutea indicating that the oocytes remaining either respond poorly to gonadotrophins or that the revascularization of the graft was inadequate.

The reproductive data from the transplanted mice (that is young ovary into aged recipient and aged ovary into young recipient) suggest that both the aged uterus and the aged ovary contribute to embryonic mortality in CBA mice. The relatively high number of resorptions in aged mice (7 of 18) receiving bilaterally or unilaterally transferred ovaries from younger mice (Table 2) suggest a uterine factor. The quality of the ova released from aged ovaries transplanted into young mice appears questionable and suggests an ovarian factor. In this group there was a small number of implantation sites and a high rate of resorptions (43%) in the uteri of young animals. None of the young mice which became pregnant after receiving a unilateral aged ovarian transplant had any implantation sites in the uterine horn adjacent to such a transplant (Table 2). Previous studies (Stevens, 1955; Krohn, 1978) have shown that the grafted ovary remains dormant in mice receiving unilateral grafts until the other ovary is removed. It appears that the non-grafted ovary has a greater gonadotrophin uptake since it exhibits some compensatory hypertrophy (Krohn, 1978).

The present research confirms the usefulness of the CBA mouse as a model to study reproductive aging. The reduction in litter size in this strain of mouse is due to age-related changes in both the uterus and ovary. The hormonal data indicate that the hypothalamic-pituitary axis is functioning adequately in all age groups examined.

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CHROMATIN CONDENSATION IN THE AGING HOUSEFLY

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INTRODUCTION

CHROMATIN IN the interphase cell nucleus can exist in a variety of condensation states. These condensation states are often divided into two broad catageries characterized by a low density or diffuse component (euchromatin) and a high density component, often referred to as heterochromatin (Brown, 1966; Grumbach, *et al.*, 1963; Hsu, 1962). Heterochromatin is further divided into constitutive and facultative heterochromatin. Constitutive heterochromatin generally consists of highly repetitive DNA sequences, is non-coding and is usually found in the centromeric regions of chromosomes. Facultative heterochromatin, however, is formed by the condensation of euchromatin during development (Lyon, 1974). Since it is difficult to distinguish between constitutive and facultative heterochromatin at the light microscope level these two types of chromatin are sometimes referred to simply as condensed chromatin (Kiefer, *et al.*, 1973; Littau, *et al.*, 1965). Euchromatin is generally found to be transcriptionally active while the activity of condensed chromatin is either nonexistent or greatly reduced (Frenster, *et al.*, 1963; Littau, *et al.*, 1964; McLean and Hilder, 1977). The correspondence between structural conformation of chromatin and transcriptional activity has also been demonstrated through ultrastructural studies (Hamkalo and Rattner, 1980) and DNAse I digestion studies (Weintraub and Groudine, 1976).

Condensation of chromatin, with subsequent reduction in transcriptional activity, has been demonstrated during the differentiation of insect flight muscle (Panar and Nair, 1975), during the maturation of various mouse cell types, including erythrocytes and goblet cells (Gersh, 1973), and during programmed cell death or apoptosis (Wyllie, *et al.*, 1981).

Chromatin condensation also appears to be associated with the aging process. Von-Hahn (1966) and Phytila and Sherman (1968) demonstrated an increase in DNA thermal stability with age when one to three percent residual histone was left intact. Purified DNA showed no such age related trend, suggesting an age related conversion to a tighter histone-DNA

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bonding pattern. Subsequently O'Meara and Hermann (1972) have shown that chromatin from the liver of old mice is more resistant to salt dissociation than liver chromatin from young mice. Transcriptional activity was shown to decrease over the same time period.

The purpose of this study was to refine the characterization of interphase chromatin condensation patterns with age and to make a detailed comparison of these patterns between nuclei from two distinct tissue types. To this end we have employed computer assisted Feulgen microspectrophotometry, whereby a digital image of a cell nucleus is generated which can be subjected to a pattern analysis after the methods of Wied, *et al.* (1968), Vidal, *et al.* (1973), and Sherwood, *et al.* (1976). In addition we have used a program, developed by one of us (Panno), which analyzes the two dimensional symmetry of the interphase condensation patterns.

MATERIALS AND METHODS

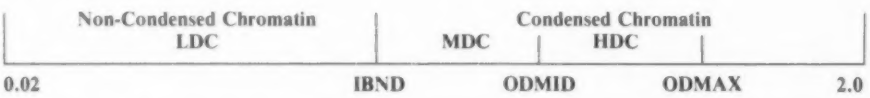
Adult houseflies were reared according to Rockstein and Lieberman (1959) on milk powder, sugar and water in 3500 cc. nylon mesh cages. The colonies were maintained in an incubator at 27°C, 40% relative humidity and a 15 hour photoperiod. Under these conditions the adult males had a mean and maximum lifespan of 14 and 28 days respectively. The mortality of the males at 14 days was 70%, which increased to 90% by day 17.

Adult males were collected, anesthetised very briefly with CO_2, and then decapitated. The heads and abdomens were fixed in bulk in buffered (pH 7.0) 10% formalin for two hours after which the tissues were washed for two hours in distilled water and then stored in 70% ETOH. Further processing occurred after all tissues from each age group had been collected. This involved acid hydrolysis in 3.5N HCL for 30 minutes at 37°C (Fand, 1972), followed by staining in fresh Schiffs reagent (Basic Fuchsin, Harleco) for one hour in the dark. The bulk-stained brain and Malpighian tubules were dissected out and cleaned of fat body and trachea. The optic lobes and the Malpighian tubules were then squashed on gelatinized glass slides, dehydrated, cleared and mounted in oil of matching refractive index (nD = 1.56, Cargille). Slide preparations were thus obtained for four individuals from each age group. Nuclei from the brain were examined from seven age groups (4 hours, 1 day, 2 days, 3 days, 4 days, 8 days and 14 days), while nuclei from the tubules were examined from four age groups (1 day, 4 days, 8 days and 14 days). We have not included age groups beyond 14 days to avoid problems of cross-sectional sampling (Sohal and Donato, 1978). Squashes were also prepared from Feulgen-stained testis to establish the ploidy levels of the brain cell nuclei and the tubule nuclei. The nuclei measured in the brain were from Type II optic lobe neurons (Sohal, 1972). In the tubules, nuclei of all classes present were measured at random. A total of 100 nuclei were measured from the brain and from the tubu'es for each age group.

All measurements were obtained with the Scanning Microscope Photometer (SMP, Carl Zeiss) which is on-line with PDP-12 and IBM-3240 computers. The diameter of the measuring aperture was 0.5 μm. The Feulgen-stained nuclei were scanned at 575 nm using a step size of 0.5 μm.

A fundamental problem in chromatin pattern analysis is that of selecting a suitable optical density (OD) that will serve as a boundary separating noncondensed chromatin (NCC) from condensed chromatin (CC) Although some authors have adopted a single boundary solution (Vidal, *et al.*, 1973; Sherwood *et al.*, 1976; Mello, 1978) we have not found it to be entirely satisfactory and instead have adopted the following procedure.

An initial boundary (IBND) separating NCC from CC was calculated by the method of Vidal, *et al.* (1973), using nuclei from the youngest age group for each tissue type. Next the average maximum optical density (ODMAX) was obtained for each cell type, and from this a midpoint (ODMID) between IBND and ODMAX was calculated. This procedure generates two major chromatin components; the non-condensed or low density component (LDC) extending from an optical density of 0.02 up to IBND, and a condensed component, extending from IBND to 2.0. The condensed component is further divided into two subcomponents; the Medium Density Component (MDC) extending from IBND to ODMID, and a High Density Component (HDC) extending from ODMID to ODMAX. The location of these components over an optical density range of 0.02 to 2.0 is diagrammed below.



The boundaries for MDC and HDC were calculated for each ploidy class. Once these boundaries were established it was possible to generate approximately 30 image features per component relating to the magnitude, spatial symmetry, and texture of the condensation process. Comparisons between the different age

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groups based on these features were analyzed for significance ($p < 0.05$) using the nonparametric Kruskal-Wallis H test (Bartels, 1979).

The features showing the most significant trends are described briefly below:

1. Total OD of the High Density Component (TOHDC). The sum of all OD values within a specified boundary interval, expressed as a percentage of total nuclear DNA (the sum of all O.D. values in the digitized image).
2. Total Area occupied by the HDC (TAHDC), expressed as a percentage of the total nuclear area.
3. Total Nuclear Area (TNA). This is calculated from the total number of OD values in the digitized image, and the known distance between each point.
4. Symmetry Index (SI). An approximately circular nucleus can be divided into four nearly equal quadrants. SI gives a measure of how evenly the condensed chromatin (either MDC or HDC) is distributed over the four quadrants. SI will range from zero (perfect asymmetry) to ten (perfect symmetry) and represents an idealized symmetry.
5. Quadrant Symmetry Index (QSI). This is an estimate of the actual number of quadrants being occupied by the condensed chromatin.
6. The Number of Condensed Chromatin Clusters (NOC). Refers to one or more OD values in the HDC or MDC range which are surrounded by LDC.
7. Cluster Distance Index (CDI). This estimates the average distance of condensed chromatin clusters from the geometric center of the nucleus and expresses it as a percentage of the nuclear radius.

RESULTS

Figure 1 shows the total OD (expressed as $\log_2$ of total OD) of the brain and tubule nuclei from four age groups. The type II neuron nuclei are diploid, whereas the tubule nuclei consist of four ploidy classes of 8C, 16C, 64C and 128C nuclei. In general the total OD and therefore the total DNA content does not change with age. Pattern analysis of the tubule nuclei showed significant changes with age in the 64C class but not in the 8C or 128C class. The 16C class was not analyzed. Therefore further reference to tubule nuclei will be understood to mean the 64C class only. In addition, reference to brain nuclei will be understood to mean the type II neuron nuclei from the optic lobes.

The total area occupied by HDC (TAHDC), increases in the brain from 9.9% in four-hour old flies to 21.8% by day 3 (Figure 2A). TAHDC increases significantly in the tubules from 3.5% at day 1 to 18.7% by day 8 (Figure 2A). Figure 2B shows the total OD of the HDC (TOHDC) as a function of age in the brain and tubule nuclei. TOHDC increases significantly in the brain from 25.7% of total DNA in the four hour group to a maximum of 48.2% by day 3. TOHDC increases significantly in the tubule nuclei as well, from 7.6% in the 1 day group to a maximum of 29.1% by day 8 (Figure 2B). TAHDC and TOHDC do not change significantly after day 3 in the brain, and after day 8 in the tubule.

The apparent effect that the HDC has on total nuclear area (TNA), is shown in Figures 2C and 2D. Here we find a significant decrease in total nuclear area in both the brain and tubule nuclei. In the brain this corresponds to the increase in the amount of HDC. The tubules, however, show an increase in the amount of HDC between day 4 and day 8 (Figures 2A, 2B), while the total nuclear area remains constant over this time period.

Two dimensional symmetry analysis of the condensation event shows that the distribution of the HDC becomes progressively symmetrical with age in both the brain and tubule nuclei (Figure 3A). The symmetry index (SI) for the four-hour-old flies increases significantly from a value of 0.75 (indicating an asymmetric distribution) to 5.0 by day 4. SI also increases significantly in the tubule from 0.27 at day 1 to a maximum of 2.26 by day 8.

The actual number of quadrants occupied by HDC (QSI) for each age group is shown in Figure 3B. The HDC in the brain nuclei is restricted to essentially one quadrant at four hours, spreading to occupy nearly three quadrants by day 4. The spread of the HDC in the

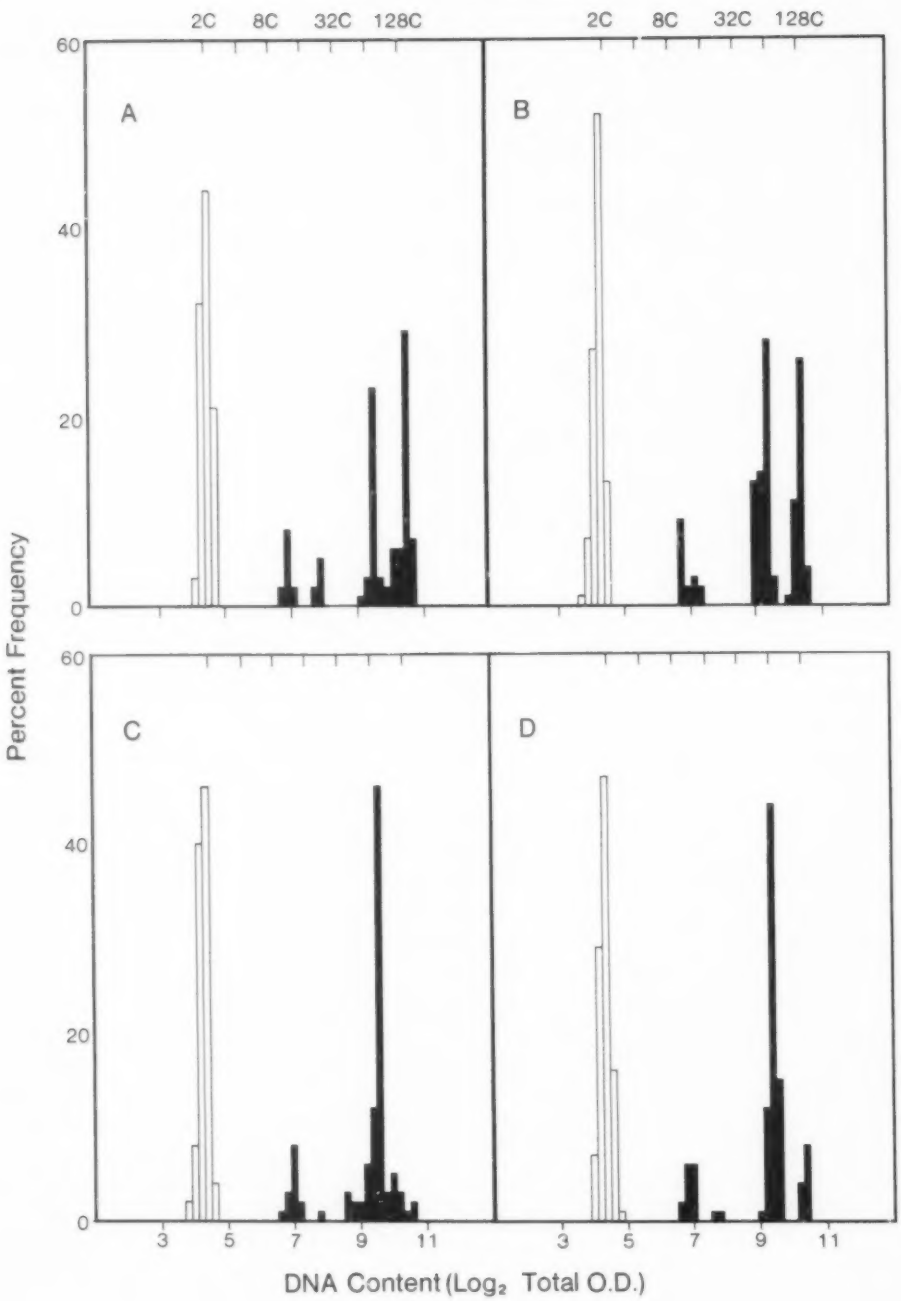

FIG. 1. Ploidy levels of the type II neuron nuclei (open histogram) and the Malpighian tubule nuclei (solid histogram) of the male housefly of 1-day(A), 4-day(B), 8-day(C) and 14-day(D) groups.

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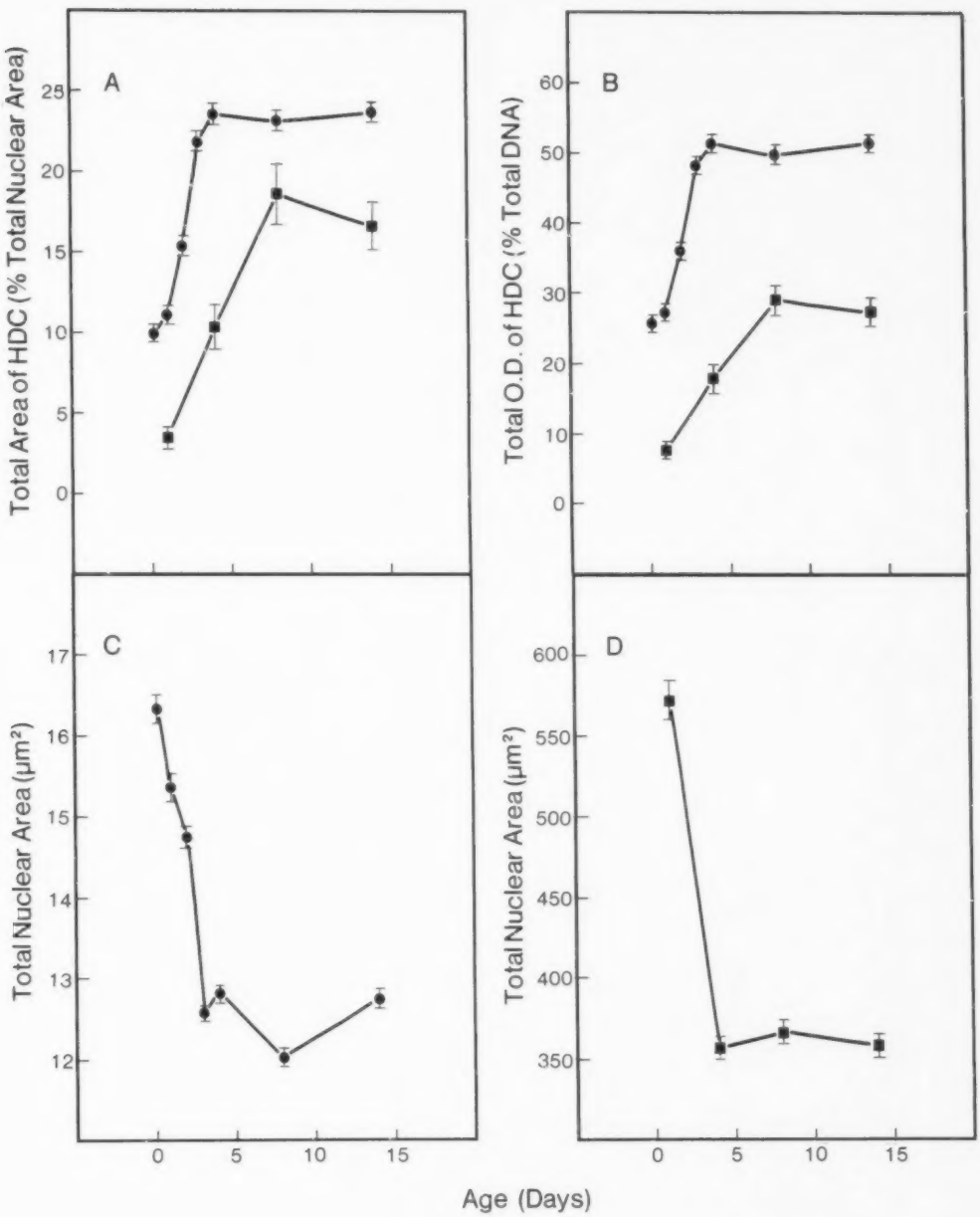

FIG. 2A. Total area of the High Density Component (HDC) as a percent of total nuclear area.
FIG. 2B. Total optical density of HDC as a percent of total DNA.
FIG. 2C. Total nuclear area of type II neuron nuclei in μm^2.
FIG. 2D. Total nuclear area of the 64C tubule nuclei in μm^2. All points represent the mean ± SE.
● Type II neuron nuclei, ■ 64C tubule nuclei.

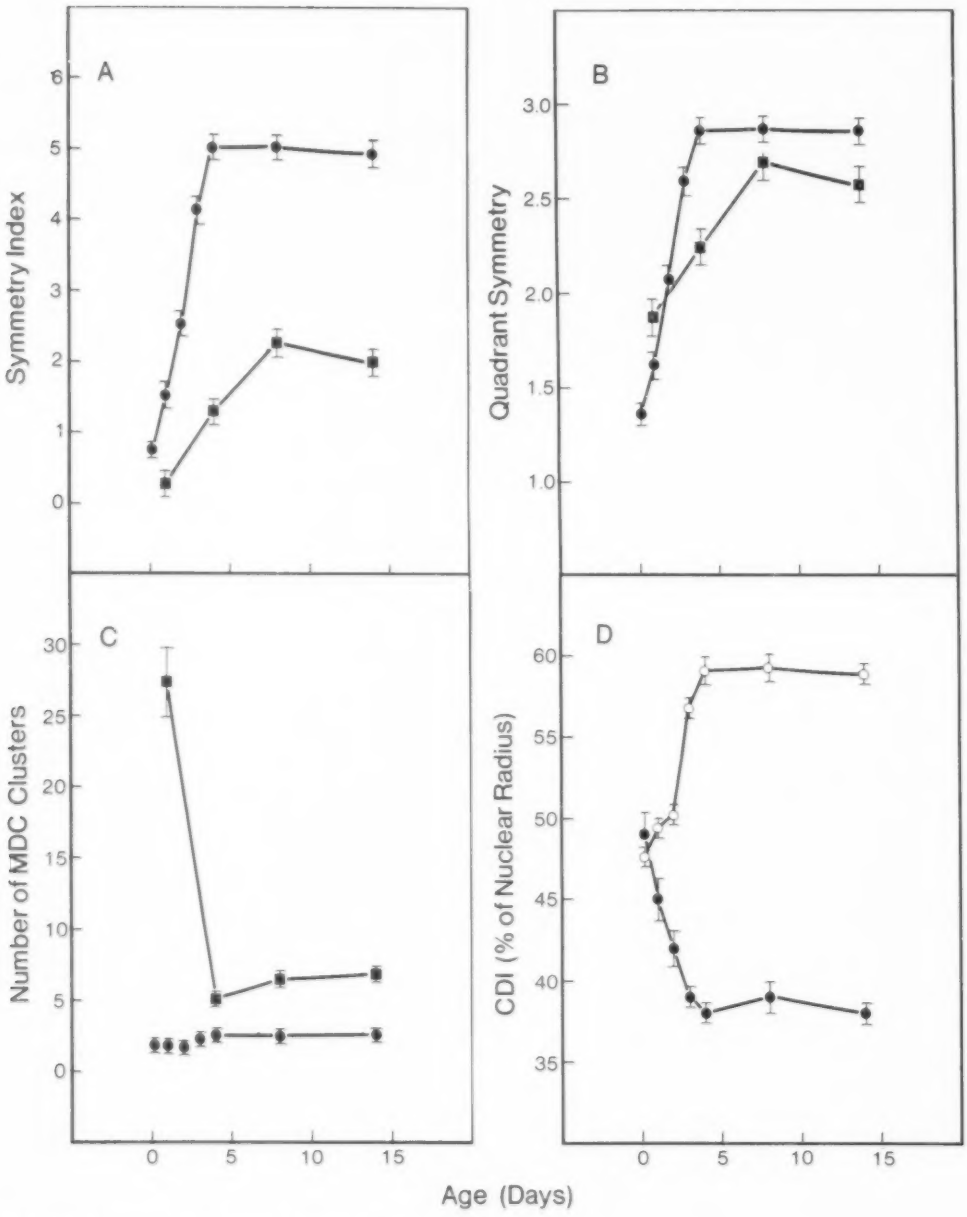

FIG. 3A. Symmetry Index as a function of age

FIG. 3B. The number of quadrants occupied by the HDC as a function of age.

FIG. 3C. The number of Medium Density Clusters (MDC) in the type II neuron and tubule nuclei as a function of age.

● Type II neuron nuclei, ■ 64C tubule nuclei.

FIG. 3D. The Cluster Distance Index (CDI) of the HDC and MDC from the type II neuron nuclei as a function of age. All points represent the mean ± SE.

○ MDC in the Type II neuron nuclei.

● HDC in the Type II neuron nuclei.

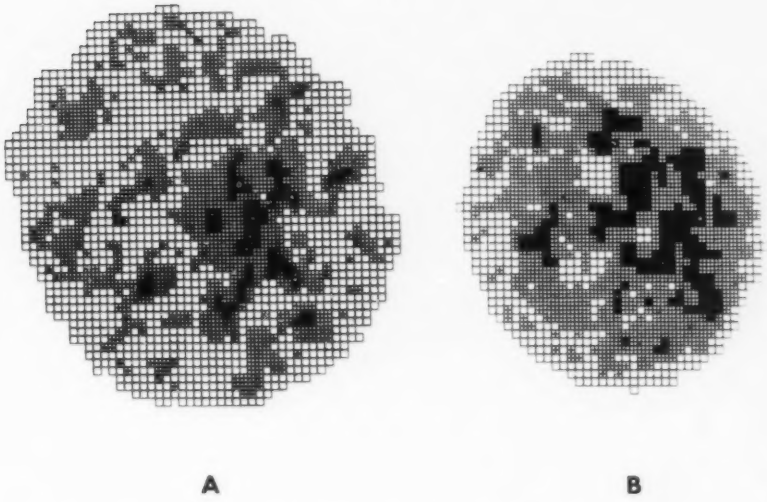

FIG. 4. Computerized images of 64C tubule nuclei which are representative of the 1-day(A) and 14-day(B) groups.
□ LDC, ⊞ MDC, ■ HDC.

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tubule nuclei is in contrast to that of the brain, in that it occupies 1.8 quadrants at day 1 and does not attain a maximum of 2.6 quadrants until day 8. SI and QSI do not change significantly after day 4 in the brain, and after day 8 in the tubule.

The HDC appears as approximately one cluster in the brain nuclei and 12 clusters in the tubule nuclei. This does not change with age. Moreover, the number of MDC clusters in the brain nuclei also remains constant with age. However, MDC appears in the tubule nuclei as 27 distinct clusters in the 1 day group, but decreases to five separate clusters by day 4, remaining constant up to day 14 (Figure 3C). Figure 3D shows the distance of the clusters from the geometric center of the nucleus (CDI). CDI of the medium and high density components in the tubule nuclei is usually about 50% of the nuclear radius and this does not change significantly with age. CDI for the MDC and HDC in the brain nuclei does show significant changes with age. The distance of the MDC clusters from the nuclear center is 48% of the nuclear radius in the 4h group and increases significantly to 59% by day 14. CDI for the HDC is 49% in the 4h group, decreasing significantly to 38% by day 14. This represents a complementary shift where the HDC displaces the MDC toward the perimeter as it moves toward the center of the nucleus. Thus a brain nucleus at day 4 can be visualized as a sphere containing a core of high density chromatin, with a surrounding shell of medium density, and possibly low density, chromatin.

Figure 4 shows computerized images of 64C tubule nuclei from the 1 day and 14 day group. These nuclei were selected by a program which evaluates all nuclei in each group with respect to how well they represent the group mean in all features considered. The general trends involving total nuclear area, MDC and HDC are clearly evident.

DISCUSSION

Pattern analysis shows significant age-related changes in the interphase chromatin condensation patterns in both the brain and tubule nuclei, while the total DNA content re-

mains unchanged. These changes relate primarily to the amount and distribution of a high density chromatin component (HDC), and to the distribution of a medium density component (MDC).

Although the brain and tubule nuclei are similar with regard to the general form of the condensation event, the results show that there are several significant differences relating to the final amount and distribution of the HDC and MDC. Perhaps the most outstanding difference between the nuclei from these two tissue types is the rate at which several features change with age. Both the condensation (TOHDC and TAHDC) and the symmetry features (SI, QSI) show the tubule nuclei changing at a significantly lower rate than the brain nuclei. The tubule nuclei consistently took from five to eight days to reach a stabilized condensation pattern while the brain nuclei took three to four days.

The difference in the rate at which the HDC in the tubules changes relative to the brain chromatin may be relevant to certain gerontological theories such as the hypothalamic elevation theory of Dilman and Anisimov (1979) or the hypothalamic disregulation theory (see review by Everitt, 1980). These theories propose that age-related changes in certain brain cells should precede senescence of other cell and tissue types, implying that an organism may be a mosaic with respect to the rate at which various cell and tissue types age. The results of pattern analysis seem to support these ideas. This pertains both to the difference between the brain and tubule nuclei and to the fact that the 8C and 128C tubule nuclei failed to show any age specific changes in the condensaton patterns.

Interpretation of these results is complicated by the fact that a number of studies have shown an eight to ten day post-emergence maturational period in a variety of dipteran insects (*Drosophila, Phormia* and *Calliphora*). However, all of these dipterans have mean lifespans nearly double that of the male housefly. In addition, the study of age-related changes in dipteran insects appears to be almost entirely concerned with changes in the flight muscle (Simon, *et al.*, 1969; Sohal, 1972; Levenbook and Williams, 1956; Sacktor and Shimada, 1972; Turturro and Shafig, 1979) or changes in whole body homogenates, much of which could be attributable to the flight muscle (Mills and Lang, 1976; Lang and Stephan, 1967). The rate at which these changes occur has been taken as the rate at which the whole organism matures and ages. We believe that this is an erroneous conclusion. If an organism constitutes an aging mosaic, then the time course of muscle development and senescence may not be the same for the organism as a whole.

Evidence for such a mosaic is apparent even in flight muscle, where the levels of cytochrome *c* increase during the first week of adulthood (Levenbook and Williams, 1956), yet the levels of isocitrate dehydrogenase in contrast to 6-phosphogluconate dehydrogenase (Lang and Stephan, 1967) and the synthetic rate of sDNA (Mills and Lang, 1976) decrease over the same time period. Similarly, Sohal (1972) has shown that the mitochondria from the flight muscle of the housefly increase in size during the first week after adult emergence, while the thoracic levels of thiamine (Rockstein and Hawkins, 1970), and the activity of acid phosphatase (Clark and Rockstein, 1964) and extra mitochondrial, NAD-dependent α-glycerophosphate dehydrogenase (Rockstein and Brandt, 1963) all decrease over this same time period.

The time course of the condensation event, particularly in the tubule nuclei, is similar to these early biochemical changes and corresponds fairly well with the peak activity period of the adult male, which extends from a few hours after emergence up to the third or fourth day. Duration of flight and wing beat frequency show a significant decline by day 5 (Rockstein and Bhatnagar, 1966). Fraying and loss of the wings characteristically begins

at about this time in the males, leaving most of them wingless by day 7 (Rockstein and Brandt, 1963). In our colonies 10% of the males had lost one or both wings by day 4, increasing to 77% by day 8. This seems to indicate that significant age-related changes have already begun to occur in these flies prior to day 4. The changing condensation patterns that we have found may be a part of these early changes.

SUMMARY

Computer analysis of digitized images of Feulgen-stained optic lobe and Malpighian tubule nuclei from the adult male housefly has shown significant age-related changes in several image features. These features relate to the amount and spatial distribution of a high and medium density chromatin component (HDC, MDC). Total DNA content in both the brain and tubule nuclei did not change with age.

The amount of HDC nearly doubles by day 3 (post-eclosion) in the brain, while the amount in the tubules increases nearly four-fold by day 8. This condensation event is accompanied by a seven- to eight-fold increase in the symmetry of the HDC distribution pattern. The number of MDC clusters in the tubule shows a five-fold decrease, while the number of MDC clusters in the brain remains unchanged.

The condensation pattern in the brain nuclei changed more rapidly than it did in the tubule nuclei. The changes in the brain nuclei were found to occur between day 1 and day 4, while the tubule nuclei showed significant changes between day 1 and day 8. The significance of this difference is discussed with respect to Hypothalamic theories on the aging process in animals.

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AGE DEPENDENT CHANGES ON JEJUNAL SUGAR ABSORPTION BY RAT *IN VIVO*

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(*Received* 7 *February* 1983)

Abstract—Jejunal absorption of D-Glucose, D-Galactose and L-Arabinose was studied in 21–23 day, 2–3 month and 18 month-old rats for 5 minute periods using a perfusion technique. Sugar concentrations were: 2.5, 5, 10, 20 and 40 mM at each age. In some experiments phlorizin was used to determine the diffusional component of absorption. The saturation for D-Glucose was at 10 mM and 5 mM for D-Galactose. A reduction in the rate of total sugar absorption was observed with aging. Moreover, the active transport/simple diffusion ratio also decreases with age, specially at low substrate concentrations.

MATERIALS AND METHODS

The experiments were carried out on male and female Wistar rats divided into three age groups: 21–23 days, 2–3 months, and 18 months old. All rats initially fasted for 18 hours. After the animals were anesthetized with urethane (12.5%) at 10 ml/Kg a laparotomy was performed and a section of jejunum was isolated between two glass canulae connected to a perfusion system equipped with a peristaltic pump (Ponz *et al.*, 1979). After rinsing with 0.9% NaCl, the intestine was perfused with 0.9% NaCl containing the sugars under study: D-Glucose, D-Galactose and L-Arabinose at 2.5, 5, 10, 20 and 40 mM concentrations. In some experiments phlorizin (5×10^{-4}M) was added to the test solution.

The solutions were recycled with a perfusion rate of 3.5 ml/min. Sugars absorption was estimated as the difference between the sugar present in the solution before and after the intestinal perfusion and expressed as μmols absorbed/cm^2 of the jejunal segment perfused during a five minute period.

Sugar concentrations were measured following a chemical method (Nelson, 1944, Somogyi, 1945).

RESULTS

Figures 1–6 show the jejunal absorption of D-Glucose and D-Galactose plotted against their initial concentrations in the perfusion solution, at different stages of life. In the presence of phlorizin (5×10^{-4}M) it was possible to determine the diffusion curve and to calculate the active transport from the difference between total absorption and diffusion.

The animals showed the smallest absorption in the old group. In order to determine if the differences observed at different stages of life are present only with the active transported sugars or also appear with the diffusionable sugars, we studied jejunal absorption of L-Arabinose in the three age groups (Figure 7).

Table 1 shows the diffusion coefficient (K_D) for D-Glucose, D-Galactose and L-Arabinose in animals of 21–23 days, 2–3 months and 18 months old, stimated from the slope of

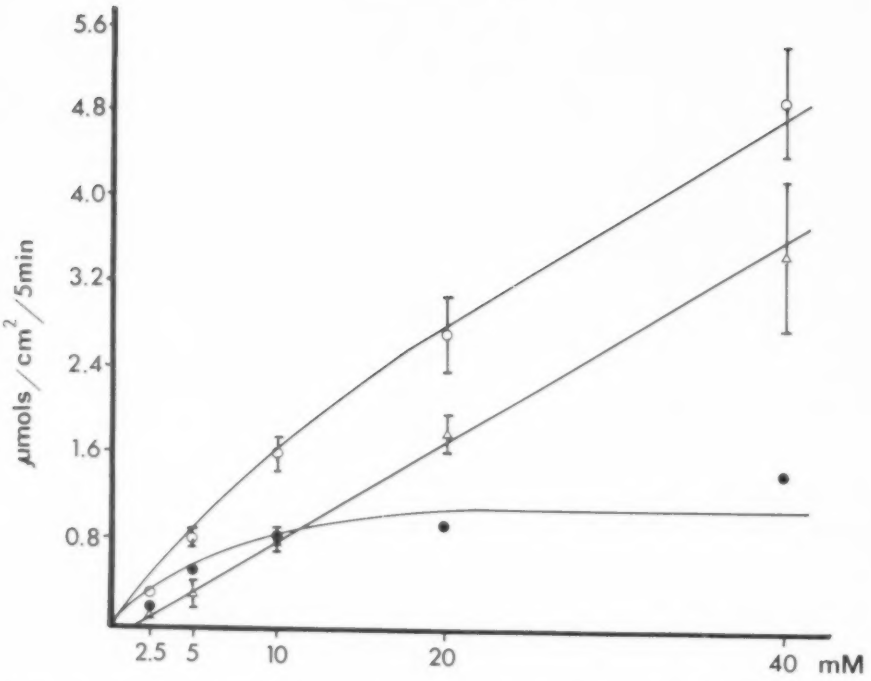

FIG. 1. Kinetics of D-Glucose jejunal absorption by 21-23 days old rats *in vivo*. (○) total absorption, (△) diffusion, (●) active transport. Mean values ± S.E.M. of at least 6 animals.

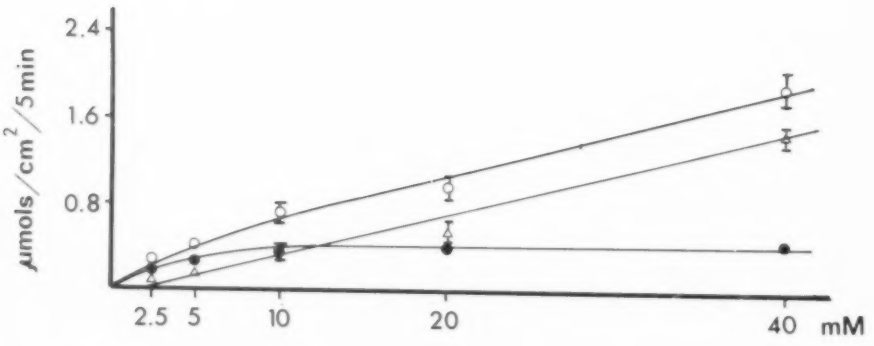

FIG. 2. Kinetics of D-Glucose jejunal absorption by 2-3 months old rats *in vivo*. (○) total absorption, (△) diffusion, (●) active transport. Mean values ± S.E.M. of at least 6 animals.

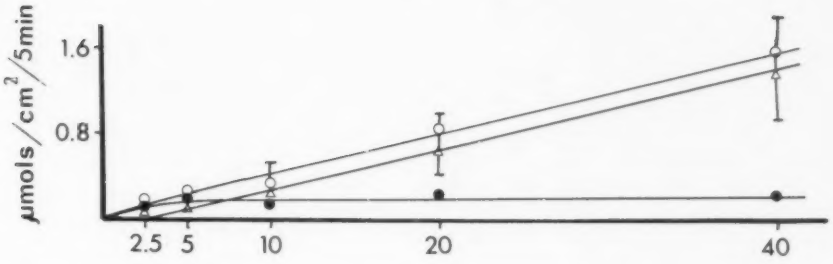

FIG. 3. Kinetics of D-Glucose jejunal absorption by 18 months old rats *in vivo*. (○) total absorption, (△) diffusion, (●) active transport. Mean values ± S.E.M. of at least 6 animals.

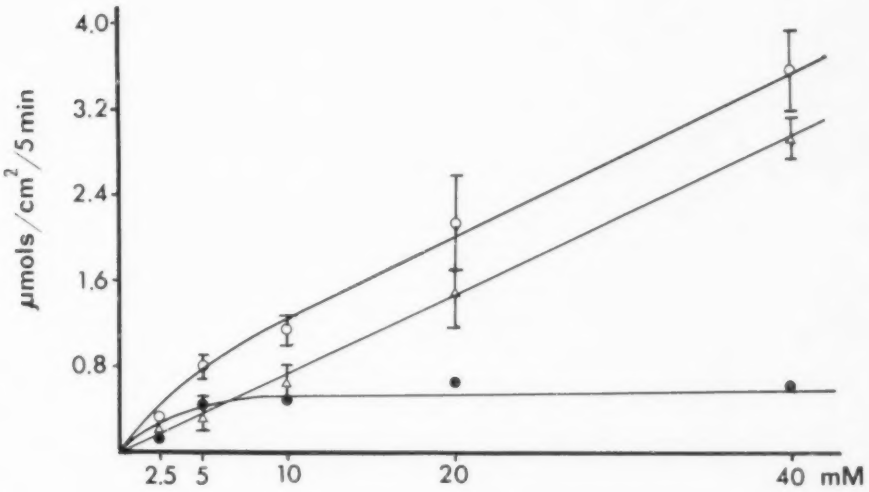

FIG. 4. Kinetics of D-Galactose jejunal absorption by 21–23 days old rats *in vivo*. (○) total absorption, (△) diffusion, (●) active transport. Mean values ± S.E.M. of at least 6 animals.

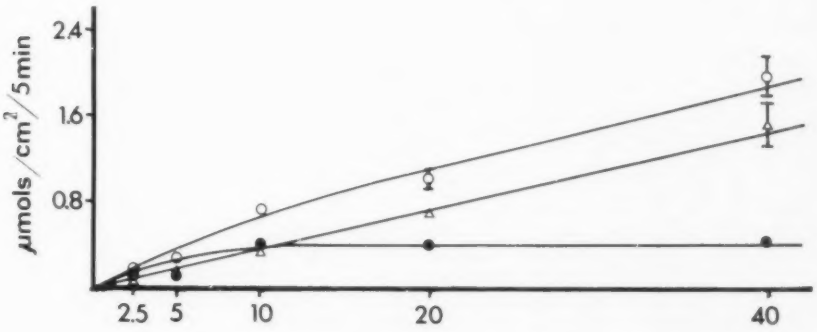

FIG. 5. Kinetics of D-Galactose jejunal absorption by 2–3 months old rats *in vivo*. (○) total absorption, (△) diffusion, (●) active transport. Mean values ± S.E.M. of at least 6 animals.

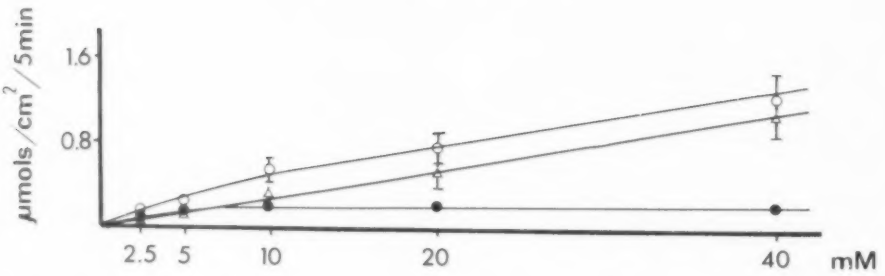

FIG. 6. Kinetics of D-Galactose jejunal absorption by 18 months old rats *in vivo*. (○) total absorption, (△) diffusion, (●) active transport. Mean values ± S.E.M. of at least 6 animals.

TABLE 1. JEJUNAL SUGARS DIFFUSION AT DIFFERENT AGES IN THE RAT

Sugar	*Age*	K_D*	*r***
D-Glucose	21–23 days	0.0907	0.9994
	2–3 months	0.0356	0.9902
	18 months	0.0358	0.9969
D-Galactose	21–23 days	0.0744	0.9910
	2–3 months	0.0395	0.9986
	18 months	0.0254	0.9973
L-Arabinose	21–23 days	0.0921	0.9991
	2–3 months	0.0276	0.9953
	18 months	0.0361	0.9911

*K_D: diffusion coefficient
**r: regression coefficient

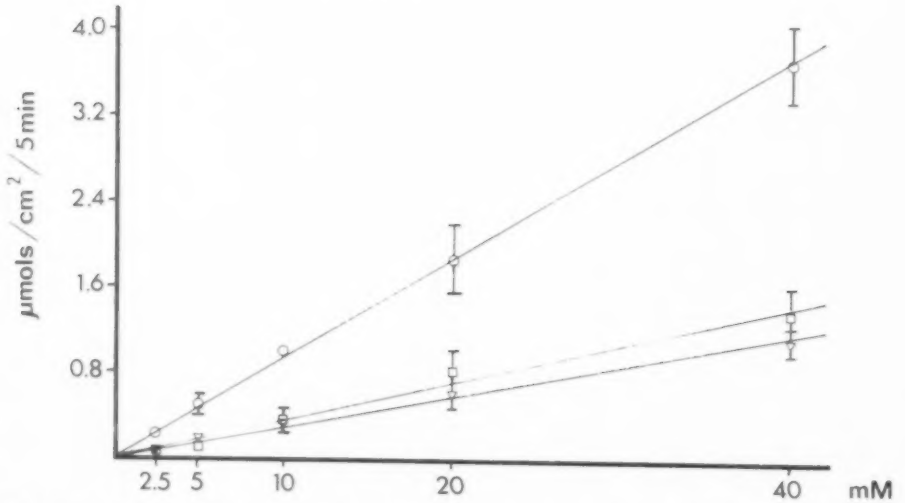

FIG. 7. Jejunal diffusion of L-Arabinose by rats of different ages *in vivo*. (○) 21–23 days, (▽) 2–3 months, (□) 18 months. Mean values ± S.E.M. of at least 6 animals.

the line that represents the diffusion absorption plotted against their initial concentration in the lumen.

DISCUSSION

As Ilundain *et al.* (1979), we observed that the intestinal absorption of D-Glucose and D-Galactose plotted against their concentration in the perfusion solution did not yield saturation curves. This was observed in all three groups studied, although the youngest animals showed the greatest absorption.

At low luminal sugar concentration the curves had two components: the active mechanism and diffusion. At higher concentrations a saturation in the jejunal absorption of D-Glucose and D-Galactose was observed. For D-Glucose the saturation corresponded to 10 mM, and the relation between the active transport and diffusion decreased with aging (active transport/diffusion: 0.99, 0.97 and 0.57 at 21–23 days, 2–3 months, and 18 months old, respectively). For D-Galactose this phenomenon was observed at 5 mM and the relation between active transport and diffusion was: 1.29, 0.85 and 0.77 at 21–23 days, 2–3 months, and 18 months, respectively.

This leads to the assumption that the diffusion plays an important role at high concentrations, especially *in vivo,* and that the active mechanism is necessary to absorb lower concentrations (Robinson and Antonioli, 1980). We obtained an active transport/diffusion relation of D-Glucose at 40 mM of 0.41, 0.31 and 0.14 for 21–23 days, 2–3 months and 18 months. In the case of D-Galactose the relation was 0.22, 0.27 and 0.18.

Our results are in accordance with other authors who found *in vivo* a decrease of the apparent passive permeability coefficient of D-Glucose with increasing age using rabbit jejunum (Thomson, 1980). We conclude that this finding is independent of the animal and the sugar employed.

The most likely reason for reduced intestinal sugar uptake in aging is that the mucosal area available for absorption is less than in young adult rats (Hohn *et al.* 1978) and similar differences have been described in men (Webster and Leeming, 1975).

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SUMMARY

Jejunal absorption of D-Glucose, D-Galactose and L-Arabinose was determined at different stages of life in the rat, using a perfusion system *in vivo*.

In all the cases there was a reduction of intestinal transport with the developing of the animal, especially in active transport.

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AN IMPROVED COLD STRESS TEST FOR AGING MICE

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(*Received* 28 *February* 1983)

Abstract—Tolerance to cold stress is a simple and reliable test which can be used longitudinally to study age-related differences or changes in thermoregulation of C57BL/6J mice. Neither prior food deprivation nor permanently inserted thermoprobes are necessary conditions for eliciting reliable age differences. However, restraint during the test is important and does elicit reliable age differences.

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INTRODUCTION

VERY FEW tests exist in experimental gerontology that can completely satisfy the criteria established at the Conference on Nonlethal Biological Markers of Physiological Aging (1981): namely, nonlethal, reproducible, and displaying age-specific alterations. Recently we confirmed an earlier observation (Eleftheriou, 1975) that the rectal temperature of male, C57BL/6J mice declines as a function of age; and we showed that this decline is not linear but begins after the age of 23.5 months (Talan, 1984). Finch (1971) has proposed that aging is not a deterioration of one or few physiological functions, but rather it is a decrease in the ability to adapt to environmental stress. In this sense, we suggest that a more appropriate measure of age change than body temperature would be the thermoregulatory response to cold stress. Grad and Kral (1957) demonstrated a marked difference between young and old female C57BL mice in the ability to withstand cold stress: that is, 73% of young mice survived 14 days exposure to a temperature of 6°–7°C while only 17% of a group of 19 month old animals did so. These findings were confirmed by Trujillo *et al.* (1962) for female RF mice and by Kiang-Ulrich and Horvath (1979) for rats. However, this technique is limited because it uses the rate of mortality as the criterion. The method described in Segall and Timiras (1975) which measures the time needed to restore body temperature following immersion of the animal in ice water for 3 minutes is one alternative. A less severe and possibly less harmful procedure was described by Verzar (1960). In his experiment rats were exposed to −4.5°–7.5°C for 1 hour during which the rectal temperature of young animals decreased 1°C, while the rectal temperature of 26-month old animals dropped by 4°C. Estler (1971) described a similar result for mice (NMRI, female) under slightly different conditions.

In a technique developed by Finch *et al.* (1969) male C57BL/6J mice were fasted for 20

hours and then placed individually in prechilled two-quart glass jars and exposed to 10°C air temperature for three hours. Colonic temperature (T_c) of 30-month old mice dropped as much as 10°C while T_c of 10-month old animals changed only slightly.

In a pilot study we were unable to reproduce the effect described by Finch *et al.* (1969). We found that T_c of old mice dropped more than that of young mice, but that there was considerable variability in the results. One explanation of the variability was that there was considerable motor activity of the mice in the relatively large experimental space. Thus, to make the technique more uniform across animals, we employed physical restraint which prevented the animals from moving excessively. Under this condition our results were almost identical to those described by Finch *et al.* (1969).

In our pilot experiment the mice were exposed to a number of conditions: 20 hour food deprivation, three hour physical restriction, three hour exposure to the low temperature and repeated measurement of T_c. The present experiments were conducted to determine the significance of each of these factors and to develop a convenient test of thermoregulatory abilities.

The objective of Experiment 1 was to assess age differences in cold tolerance when gross motor restraint was imposed. Experiment 2 was designed to detect the relative contribution and possible interaction of food deprivation and exposure to cold on thermoregulatory ability. Experiment 3 was performed to determine whether the method of measuring T_c during the cold stress test (thermoprobe permanently inserted in the colon during experiment versus repeated insertions of thermoprobe for every measure) was important to the results. On the basis of these experiments, a test protocol and calculation procedure was developed for detecting age-related differences in the ability of mice to withstand cold stress. Experiment 4 was a demonstration of age-related differences in cold tolerance performed according to this protocol.

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MATERIALS AND METHODS

Subjects

A total of 84 male C57BL/6J mice from two age groups (adult: 10-months and aged: 30-months) were obtained from the animal colony of the Gerontology Research Center*. Sixteen were designated for Experiment 2 (eight adult and eight aged) and these were housed individually in metal cages with water and food available *ad libitum*, except during the experimental treatment. The remaining animals (16 adult and 22 aged for Experiment 1; 7 adult and 7 aged for Experiment 3; and 8 adult and 8 aged for Experiment 4) were housed in plastic cages with sawdust bedding, four to five per cage, with food and water available *ad libitum*. Temperature in the room was controlled automatically at a level of 22.5°C ± 0.5°C and a photoperiod of 12-hours light: 12-hours dark was maintained.

General procedures

Motor restraint Before every cold stress test, each animal was placed into a specially designed restrainer. It consisted of a Plexiglas® tube, 10 cm long and 3 cm in internal diameter. Four to six holes of 1 cm diameter each were made to allow circulation of air. The ends of the tubes were closed by rubber stoppers. One stopper had a V-shaped slot for the animal's tail. Each restrainer was placed onto a special holder designed to maintain air circulation around it.

Temperature recording Prelubricated Yellow Spring Thermoprobes (Series 500) were inserted 2–3 cm into the

*The Gerontology Research Center is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

colon and fixed to the tail with tape. The probes remained fixed in this position during the entire 3-hour period in Experiment 2 and partly in experiment 3, or inserted every 30-min during the test in Experiment 1,4, and partly in 3. T_c was measured by using a telethermometer (Yellow Spring Instruments). In the experiments when the thermoprobes were inserted each time, 10–15 seconds elapsed before T_c was read.

Cold stress tests After the thermoprobes were inserted or the first temperature reading was taken, restrained animals were moved into a "walk in" cold room maintained at $+10°C \pm 0.5°C$. Animals were kept in the cold room 180 minutes during which T_c was recorded every 30 minutes. However, if T_c dropped below 24°C, the test was terminated. After the test all mice were warmed under a heat lamp for one hour before they were returned to their home cages.

Statistical analysis The slope of T_c versus time was calculated for each animal. These slopes were then submitted to an analysis of variance (ANOVA). All figures presented below reflect the mean T_c change for each group calculated on the bases of the mean slopes and intercepts.

Experiment 1

Two age groups were used (16 adult and 22 aged) and these groups were divided evenly into two experimental groups. Animals from group 1 were subjected to a cold stress test similar to that described in Finch *et al.* (1969). They were placed into the cold room in prechilled empty plastic cages 30 × 17 × 12 cm, one animal per cage. T_c was recorded immediately after placement into the cold room and then subsequently every 30 minutes. Animals from group 2 were placed into restrainers followed by exposure to the cold. They stayed in the restrainers during the entire experiment. Animals from both groups were fasted for 20 hours before testing.

Experiment 2

Each of eight adult and eight aged mice was tested in four different conditions at one-week intervals. For each test, animals were placed into restrainers with thermoprobes inserted into the colon and T_c was taken every thirty minutes during a three-hour period. These four conditions were: restraint for three hours at room temperature (R); restraint at room temperature following 20 hours of food deprivation ($\bar{F}$R); restraint for three hours in the cold room with ambient temperature 10°C (CR); restraint for three hours in the cold room following 20 hours of food deprivation ($\bar{F}$CR). To control the influence of preceding test conditions on the results of subsequent tests, sequences of experimental conditions for each animal were counterbalanced using a latin square design.

Experiment 3

Aged mice were randomly divided into two groups, seven animals each. All were tested twice in the cold stress at one-week intervals. Preliminary food deprivation was not applied in this experiment. In the first test animals from group 1 had thermoprobes inserted into the colon before the test and kept inserted for 180 minutes while animals in group 2 had thermoprobes inserted repeatedly for each T_c reading. For the second test, the conditions were reversed with the animals from group 2 having the thermoprobes fixed throughout the test.

Experiment 4

Experiment 4 will be described later.

RESULTS

The results of Experiment 1 are presented in Figure 1. An age difference in response to cold stress was observed only in the restrained conditions. The results of ANOVA revealed a significant main effect for age ($F(1,34) = 22.48$, $p < 0.001$) and condition of experiment (unrestrained animal versus restrained) ($F(1,34) = 9.55$, $p < 0.01$). There was also a significant interaction between age and conditions, ($F(1,34) = 17.68$, $p < 0.001$). Analysis of the simple main effects (Winer, 1971) demonstrated that older animals were more impaired than were young animals only under the restrained conditions during cold stress, ($F(1,34) = 36.03$, $p < 0.001$). Therefore, motor restraint appears to be an important factor for eliciting age-related differences among food deprived mice during cold stress.

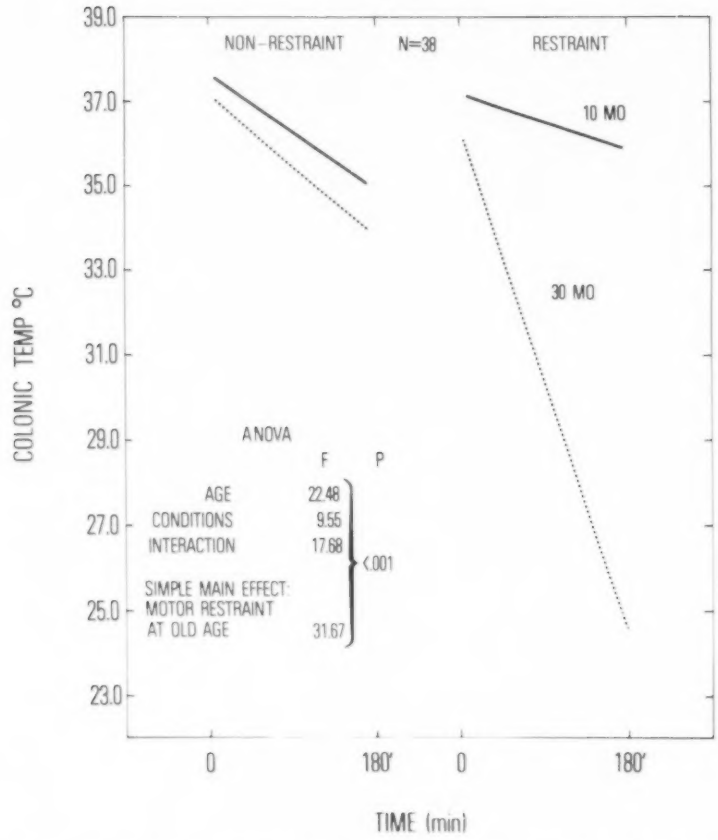

FIG. 1. Results of the cold stress test for 10 month old and 30 month old mice after 20 hours food deprivation during unrestrained (left part) and restrained (right part) motor activity.

Figure 2 illustrates the results of Experiment 2. Preliminary food deprivation ($\bar{F}$) for 20 hours is associated with decreasing T_c of old mice to a greater degree than among adult animals. Restraint during three hours at room temperature (R) resulted in approximately equivalent decreases in T_c among adult and old animals. Preliminary food deprivation increases T_c decline of old animals during three hours restraint at room temperature ($\bar{F}R$), but the combination of these treatments did not affect T_c in adult mice beyond that attributable to restraint alone. The condition referred to as RC (three hours motor restraint during exposure to 10°C of ambient temperature) resulted in substantial differences in responses between adult and old animals. The age difference was even more apparent after 20-hours food deprivation ($\bar{F}CR$).

These conclusions were substantiated by the results of ANOVA, which revealed a significant main effect of age ($F(1,14) = 18.17$, $p < 0.01$); a significant main effect of the cold exposure ($F(1,14) = 23.32$, $p < 0.01$); and a significant main effect of preliminary food deprivation, ($F(1,14) = 11.13$, $p < 0.01$). There was no significant interaction between food deprivation and cold exposure ($F(1,14) = 1.61$, $p > 0.1$); however, there was a

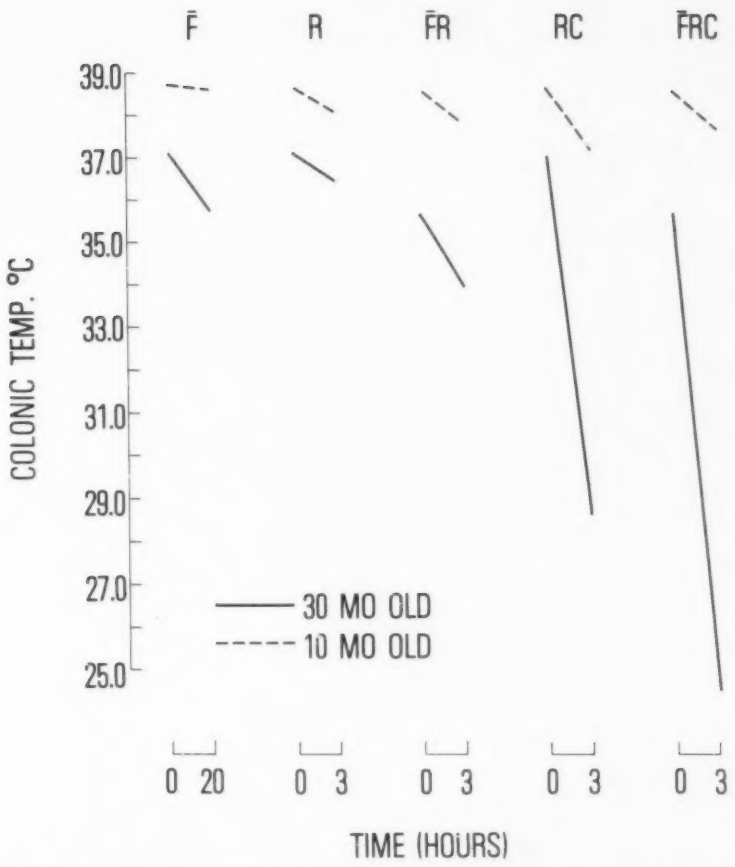

FIG 2. Changes the body temperature of 10 month old (dotted lines) and 30 month old (solid lines) mice after 20 hours food deprivation ($\bar{F}$) and four different 3-hour experimental conditions. See text for description.

significant interaction between age and cold exposure ($F(1,14) = 18.26, p < 0.01$). Tests of simple main effects indicate that a significant effect of cold exposure was observed only among the old animals ($F(1,14) = 41.45, p < 0.001$) while the cold exposure did not affect adult animals in this experiment ($F(1,14) = 0.15, p > 0.1$). In addition, there was a significant interaction between age and pre-test food deprivation ($F(1,14) = 11.65$, $p < 0.01$). Analysis of simple main effects indicated that the responses were affected by food deprivation only among old animals ($F(1,14) = 24.32, p < 0.01$) and not among adults, ($F(1,14) = 1.99, p > 0.1$). Therefore, food deprivation was not critical for demonstration of age-related differences during the cold stress test: these differences were revealed by exposure to cold alone.

Experiment 3 evaluated the method used to measure temperature. Figure 3 illustrates the results of this experiment which indicated that the method of T_c recording did not affect the results of the cold stress test, that is, there were no significant main effects for groups or tests and no significant interactions.

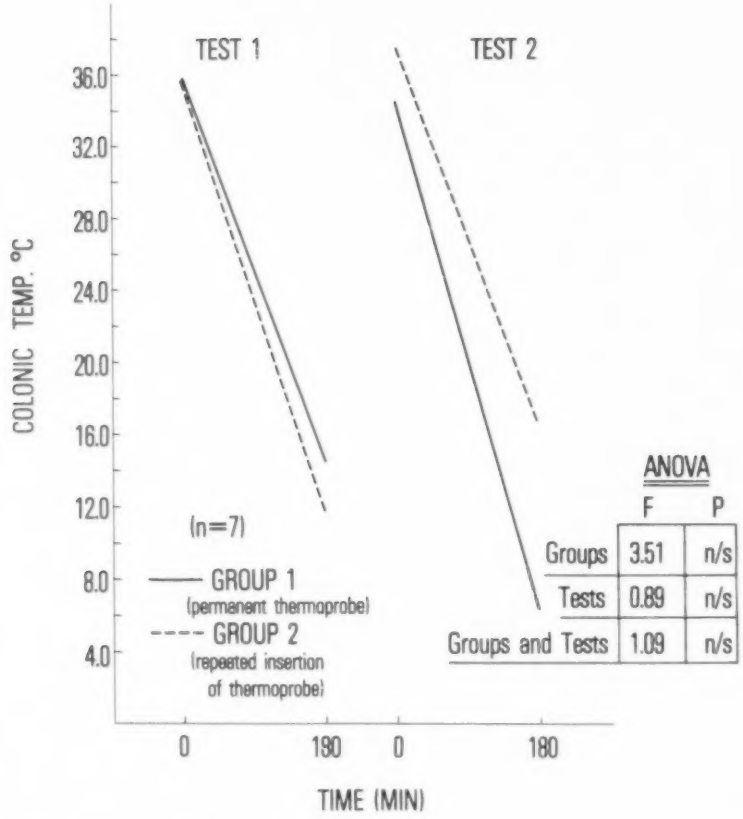

FIG. 3. Results of cold stress test for 30 month old mice using permanent thermoprobe during the test or repeated insertion of thermoprobe. Solid lines – permanent thermoprobe in test 1 or repeated insertion in test 2; Dotted lines – repeated insertion of thermoprobe in test 1 or permanent in test 2. The time between two tests is 1 week.

The first three experiments demonstrated that in order to observe differences in tolerance to cold stress between adult and old mice, animals should be restrained during the test but need not be food deprived before testing. Repeated insertion of the thermoprobe into the rectum during testing did not affect significantly the results of the cold stress test. Experiment 4 was performed to characterize the differences between old and adult mice in their response to cold stress. In this experiment animals were not food deprived, but were restrained for three hours and T_c was measured by insertion of the thermoprobe every 30 minutes. All animals from both groups completed the experiment except one mouse from the 30 month old group in which T_c decreased below 24°C after 90 minutes of cold exposure. The mean T_c decline was markedly steeper for old mice (Figure 4); the results of the ANOVA of individual slopes demonstrated a significant difference between the two age groups ($F(1,14) = 28.29, p < 0.001$). Figure 4 also gives the regression coefficients and intercepts for the two age groups.

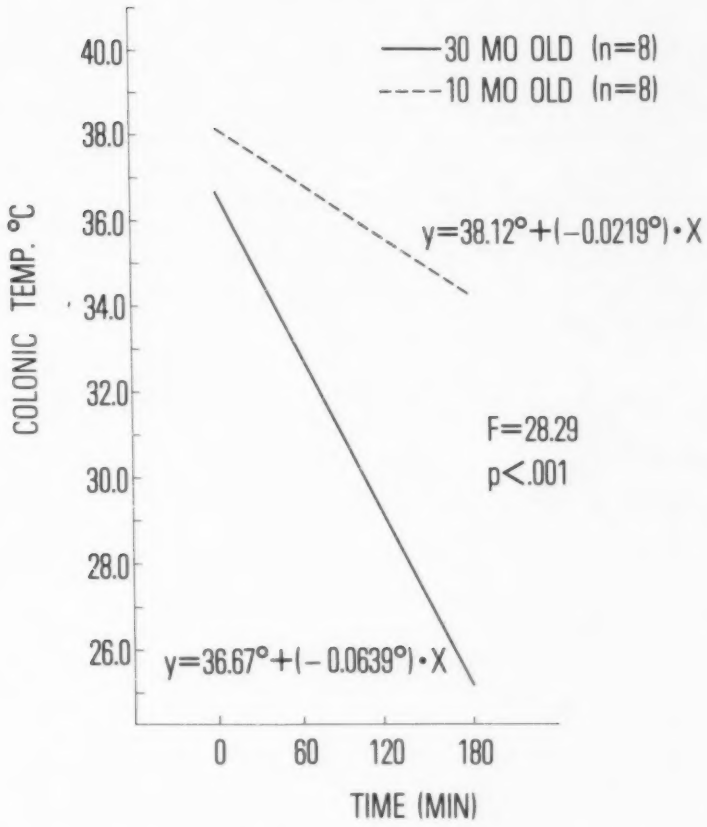

FIG. 4. Results of cold stress test (according the protocol) for 10 month old (dotted line) and 30 month old (solid line) mice.

DISCUSSION

Past reports and pilot observations in this laboratory suggested that tolerance to cold stress might be a reliable test of age-related differences in thermoregulation of mice. The cold stress test described by Finch *et al.* (1969) was selected for evaluation as particularly suitable for gerontological research. However, the results described in Finch *et al.* (1969) were not replicated using the protocol of their experiments. Although we were unable to replicate their findings in animals allowed relatively unrestricted activity, when motor restraint was added to the protocol the results were replicated.

The objective of the present study was to develop an appropriate and reliable protocol for a cold stress test that could be applied longitudinally using C57BL/6J mice. Several variables were studied to isolate the major factors. Thus, motor restraint during cold stress test is an important factor for observing an age difference. However, preliminary food deprivation for 20 hours was not an important variable since no interaction was

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found between exposure to cold and prior food deprivation. This finding made the cold stress test more convenient for longitudinal research since 20-hour food deprivation itself, may be life-threatening for older mice. Another finding was that the thermoprobe could be inserted periodically during the cold stress test, a cost-effective convenience which allows large numbers of animals to be evaluated at the same time.

Based on these findings, the following protocol for a cold stress test for C57BL/6J mice was developed: The animal is restrained in a Plexiglas® cylinder, 3 cm in diameter and 10 cm in length, with multiple holes for access to air; it is exposed to a temperature of 10°C for 3 hours (unless T_c falls below 24°C); T_c (insertion of the thermoprobe is about 2–2.5 cm) is taken initially and at 30 minute intervals); after testing the animals are placed under a warming lamp for 1 hour.

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AGE RELATED EFFECTS OF NUTRITIONAL VITAMIN B_6 DEFICIENCY ON B_6-DEPENDENT ENZYMES OF GLUTAMATE, γ-AMINOBUTYRATE AND GLUTAMINE SYSTEMS IN THE RAT BRAIN

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Abstract—Effect of nutritional vitamin B_6 deficiency on the activities of enzymes-glutamic acid decarboxylase (GAD), gamma aminobutyric acid transaminase (GABA-T), aspartate aminotransferase (AsAT), alanine aminotransferase (AlAT) and glutamotransferase (GT) was studied in the rat brain of different age groups. Deficiency was induced at one day (group 1), 21 days (group 2), three months (group 3), 12 months (group 4) and 24 months (group 5) for a period of 3–10 weeks. When the holo and apoenzyme levels were compared in control and B_6 deficient rats, it was found that GAD, GABA-T, AsAT and AlAT holoenzyme levels were significantly lower in the deficient rats of all the five groups whereas the level of GT holoenzyme was low only in group 1, the other groups did not show any significant change. GAD apoenzyme level was significantly higher in the deficient animals of group 1 and 2 while the reverse was true for GABA-T apoenzyme and no significant variation was seen in the older groups. It is interesting to note that the level of AsAT apoenzyme was significantly low in older age groups (4, 5) as a function of B_6 deficiency, while AlAT and GT apoenzymes were not affected in any age group studied.

INTRODUCTION

VERY LITTLE is known about the effects of vitamin B_6 deficiency on the aged brain. However, it has been reported that 19–28% of people of 65 years and older are B_6 deficient (Hoorn *et al.*, 1975; Vir and Love, 1978). Most of the studies so far have been with reference to developmental stages (Roberts *et al.*, 1951; Bayoumi and Smith, 1972; Bayoumi *et al.*, 1972; Brin and Thiele, 1967; Thiele and Brin, 1968; Stephens *et al.*, 1971; Kurtz *et al.*, 1971). Alterations in the behavioral patterns of older animals as a function of B_6 deficiency have been reported (Gewacke *et al.*, 1977; Ingram and McDaniel, 1979). In this paper we discuss the effect of B_6 deficiency on the amino acid metabolism in the brain with reference to aging.

MATERIALS & METHODS

Vitamin B_6 deficiency was induced in albino rats (Wistar strain) of one day (group 1), 21 days (group 2), three (group 3), 12 (group 4) and 24 (group 5) months of age which were maintained at 28 ± 2°C and 12L:12D. In the

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case of group 1 the deficiency was induced by feeding the mothers for three weeks with a pyridoxine deficient diet (Bayoumi and Smith, 1972). Group 2 and 3 were on deficient diet for four weeks (Tews and Lovell, 1967; Bayoumi and Smith, 1973), while groups 4 and 5 were on the same diet for ten weeks (Gewacke *et al.*, 1977). The diet formula (Rajalakshmi *et al.*, 1974) consisted of 72% carbohydrate, 15% casein, 7% peanut oil, 4% Hawk-Oser mineral mixture (Oser, 1965) and 2% vitamin mixture. Pyridoxine HCl content was 50 μg/100 g diet (Beaton and Cheney, 1965) for the control, in the case of experimental animals it was omitted.

The animals were decapitated at the end of the experimental period and the brain was quickly removed, washed in ice cold 0.9% saline, weighed and used for assays. GAD was assayed according to Cozzani (1970) using Gabase system (Jackoby and Scott, 1959) and GABA-T by the method of Baxter and Roberts (1958). AsAT and AlAT as described by Sudgen and Newsholme (1975) and GT according to Rudnick and Waelsch (1955). The assays were performed with 50 μg/ml of homogenizing medium with and without PLP to quantify the degree of endogenous co-factor saturation of the enzyme.

RESULTS

Tables 1–3 show activities of B_6 dependent enzymes of glutamate, GABA and glutamine systems with and without exogenously added PLP in the brain of control and vitamin B_6 deficient animals as a function of age.

The holoenzyme level of GAD was significantly low in the brain of deficient animals of all the five age groups studied. Maximum effect due to deficiency was seen in the brain of group 1 showing only 15% activity while the groups 2, 3, 4 and 5 showed 38, 36, 30 and 21% of activity compared to controls. The GAD apoenzyme levels were significantly higher in the deficient groups 1 (69%) and 2 (12%) while in the older groups there was no significant difference (Figure 1).

In the control rats the activity of GABA-T holoenzyme when compared to apoenzyme was 87%, 86%, 83%, 73% and 63% in groups 1–5 respectively. In the deficient animals the level of holoenzyme was significantly lower compared to the controls in all the five age groups. The level of GABA-T apoenzyme in pyridoxine deficient group 1 was 67% of

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TABLE 1. ACTIVITY OF GLUTAMIC ACID DECARBOXYLASE AND GAMMA AMINOBUTYRIC ACID-TRANSAMINASE AS A FUNCTION OF AGE AND PYRIDOXINE DEFICIENCY IN RAT BRAIN.

	GAD activity (μmole of γ-ABA formed/g tissue/min)			
	Control		*Experimental*	
Group	*+ PLP*	*− PLP*	*+ PLP*	*− PLP*
1	2.26 ± 0.22	1.09 ± 0.11	3.81 ± 0.16	0.34 ± 0.07*
2	3.58 ± 0.24	2.20 ± 0.19	4.00 ± 0.19	1.36 ± 0.09*
3	4.23 ± 0.24	2.71 ± 0.15	4.10 ± 0.17	1.51 ± 0.10*
4	4.59 ± 0.21	3.24 ± 0.22	4.69 ± 0.15	1.37 ± 0.14*
5	5.10 ± 0.24	3.82 ± 0.24	5.15 ± 0.21	1.08 ± 0.13*
	γ-ABA-T activity (μmoles of glutamic acid formed/g tissue/min			
1	1.08 ± 0.10	0.94 ± 0.07	0.72 ± 0.08	0.40 ± 0.08*
2	1.30 ± 0.07	1.04 ± 0.06	1.01 ± 0.10	0.81 ± 0.10*
3	1.24 ± 0.05	1.03 ± 0.06	1.16 ± 0.16	0.93 ± 0.07*
4	1.02 ± 0.14	0.74 ± 0.10	1.05 ± 0.10	0.56 ± 0.11*
5	0.88 ± 0.14	0.52 ± 0.08	0.92 ± 0.09	0.43 ± 0.12*

Values are represented as mean ± SD of 4 observations.

*$p < 0.001$. (Compared to − PLP of control).

TABLE 2. ACTIVITY OF ASPARTATE AMINOTRANSFERASE AND ALANINE AMINOTRANSFERASE AS A FUNCTION OF AGE AND PYRIDOXINE DEFICIENCY IN RAT BRAIN.

	AsAT activity (μmoles substrate utilized/g tissue/min)			
	Control		*Experimental*	
Group	*+ PLP*	*− PLP*	*+ PLP*	*− PLP*
1	34.9 ± 1.70	35.7 ± 2.40	36.2 ± 2.80	14.0 ± 1.40*
2	35.2 ± 1.91	35.8 ± 2.90	34.5 ± 1.90	18.0 ± 2.60*
3	38.1 ± 1.70	38.0 ± 3.00	35.4 ± 2.20	25.4 ± 1.00*
4	41.4 ± 2.50	40.2 ± 2.80	32.2 ± 2.40	22.6 ± 2.10*
5	40.2 ± 2.80	41.3 ± 2.80	26.0 ± 1.60	22.0 ± 1.60*
	AlAT activity (μmoles substrate utilized/g tissue/min)			
1	1.15 ± 0.14	1.03 ± 0.08	1.09 ± 0.09	0.40 ± 0.07*
2	1.22 ± 0.14	1.18 ± 0.18	1.17 ± 0.07	0.52 ± 0.05*
3	1.15 ± 0.15	1.07 ± 0.13	1.21 ± 0.19	0.53 ± 0.06*
4	1.14 ± 0.09	1.05 ± 0.19	1.07 ± 0.09	0.38 ± 0.05*
5	1.17 ± 0.06	1.15 ± 0.08	1.12 ± 0.13	0.33 ± 0.03*

Values are represented as mean ± SD of 4 observations.
*$p < 0.001$. (Compared to −PLP of control).

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control ($p < 0.001$) while that of group 2 was 78% ($p < 0.01$), the other age groups were not affected.

In the case of AsAT, the exogenous PLP did not alter the activity in the control rats of all age groups thereby showing that the enzyme was completely saturated with PLP. However, in the deficient rats the level of holoenzyme was significantly lower in all the age groups studied. AsAT apoenzyme significantly decreased only in older groups (4 and 5).

B_6 deficiency did not alter the apoenzyme level of AlAT in any age group whereas the

TABLE 3. ACTIVITY OF GLUTAMOTRANSFERASE AS A FUNCTION OF AGE AND PYRIDOXINE DEFICIENCY IN RAT BRAIN.

	GT activity (μmoles of GHA formed/g tissue/min)			
	Control		*Experimental*	
Group	*+ PLP*	*− PLP*	*+ PLP*	*− PLP*
1	1.23 ± 0.14	1.16 ± 0.13	1.15 ± 0.12	0.52 ± 0.06*
2	1.66 ± 0.14	1.70 ± 0.15	1.74 ± 0.19	1.59 ± 0.18 N.S.
3	1.89 ± 0.16	1.85 ± 0.12	1.81 ± 0.14	1.88 ± 0.15 N.S.
4	2.43 ± 0.14	2.58 ± 0.18	2.35 ± 0.17	2.48 ± 0.20 N.S.
5	2.93 ± 0.15	2.98 ± 0.18	2.84 ± 0.24	2.93 ± 0.21 N.S.

Values are represented as mean ± SD of 4 observations.
*$p < 0.001$. (Compared to −PLP of control).
N.S. − not significant.

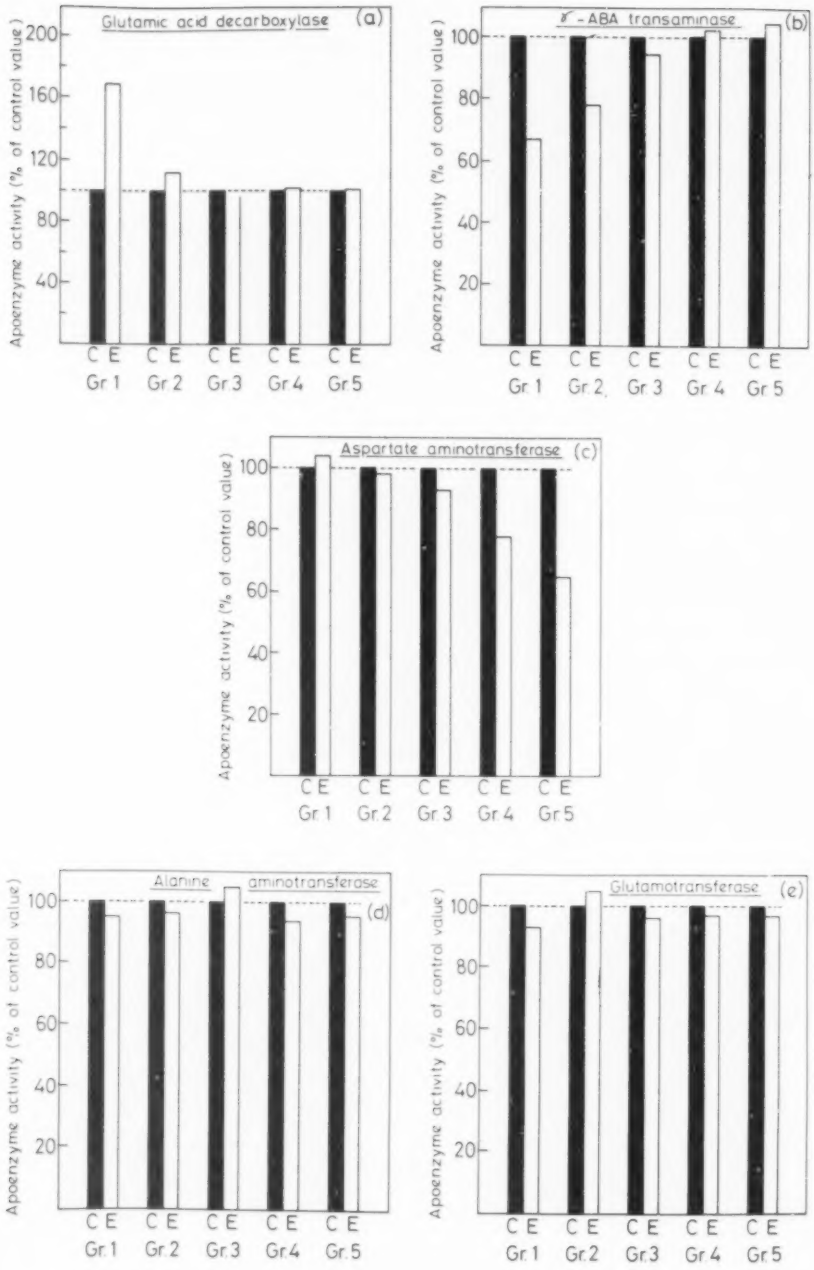

FIG. 1. GAD, GABA-T, AsAT, AlAT and GT apoenzyme activity as a function of pyridoxine deficiency at different ages in rat brain.
Gr. - Group C - Control E - Experimental

holoenzyme decreased in all the age groups. The level of holoenzyme of GT due to pyridozine deficiency was 58% in group 1, while in the other age groups the levels were not altered significantly. As in the case of AlAT the level of apoenzyme was not affected due to B_6 deficiency in any age group.

DISCUSSION

Very little is known about vitamin B_6 metabolism as a function of age. However, it has been indicated that the mechanism by which B_6 deficiency contributes to impaired brain function might be related to disturbed amino acid pool (Strecker, 1970; Tews and Lovell, 1969) and GABA metabolism since the GABA synthesizing enzyme GAD and the degrading enzyme GABA-T are B_6 dependent (Baxter and Roberts, 1958). The resistance of brain aspartate amino transferase to pyridoxine deficiency might be of functional significance due to the excitatory neurotransmitter role of aspartate and glutamate.

Our results have shown that at physiological levels of tissue B_6, only 64% of the GAD apoenzyme is in the holoenzyme form as seen in the young adult. The significant increase seen in the GAD holoenzyme of older age groups (4 and 5) can be due to decreased levels of GABA with advance in age. The activity of GAD assayed without exogenous PLP which is indicative of the endogenous saturation of the enzyme with PLP is only 48% in group 2 (Figure 2) and adult levels are reached only around three months. This may be attributed to the availability of the cofactor which has been shown to attain the adult level around three months (Sturman and Rivlin, 1975). These factors would account for the maximum rate of increase of GAD activity between 21 days and three months of age which parallels with the increase in GABA (Sturman and Rivlin, 1975). The present results have shown that the level of apoenzyme in the brain is not significantly altered due to B_6 deficiency in groups, 3, 4 and 5 and it is only the degree of saturation of the enzyme with the coenzyme that is significantly decreased. Fonda *et al.*, (1980) have shown the steady state concentrations of the coenzymes and the activities of the enzymes required for pyridoxal-P synthesis did not change significantly as a function of age. The activity of GABA-T is affected to a lesser degree than that of GAD in all the age groups studied. B_6 deficiency resulted in significant decrease in the level of holoenzyme in all the age groups, however, the effect is more in the early age groups which support the findings of Bayoumi and Smith (1972). In all the age groups the alanine enzyme is more sensitive to B_6 deficiency than that of aspartate.

The enzyme glutamotransferase like AsAT and AlAT is fully saturated with the coenzyme in the brain of control rats of all age groups. In the case of B_6 deficiency only in the developing brain the level of holoenzyme is reduced to 43% compared to the control. This may be due to inadequate binding of coenzyme to apoenzyme and has support in the studies of Cooper and Meister (1972).

B_6 deficiency leads to decrease in the coenzyme (Stephens *et al.*, 1971; Barker and Bender, 1980) and results in significant decrease in the activity of most of the B_6 dependent enzymes, but the degree varies with age. It was also noted that the transaminases are less susceptible to deficiency than the decarboxylases. This differential susceptibility would reflect weaker PLP-apoenzyme binding in the decarboxylase system (Scriver and Hutchinson, 1963).

From the present results it is clear that the dietary B_6 deficiency would affect certain of

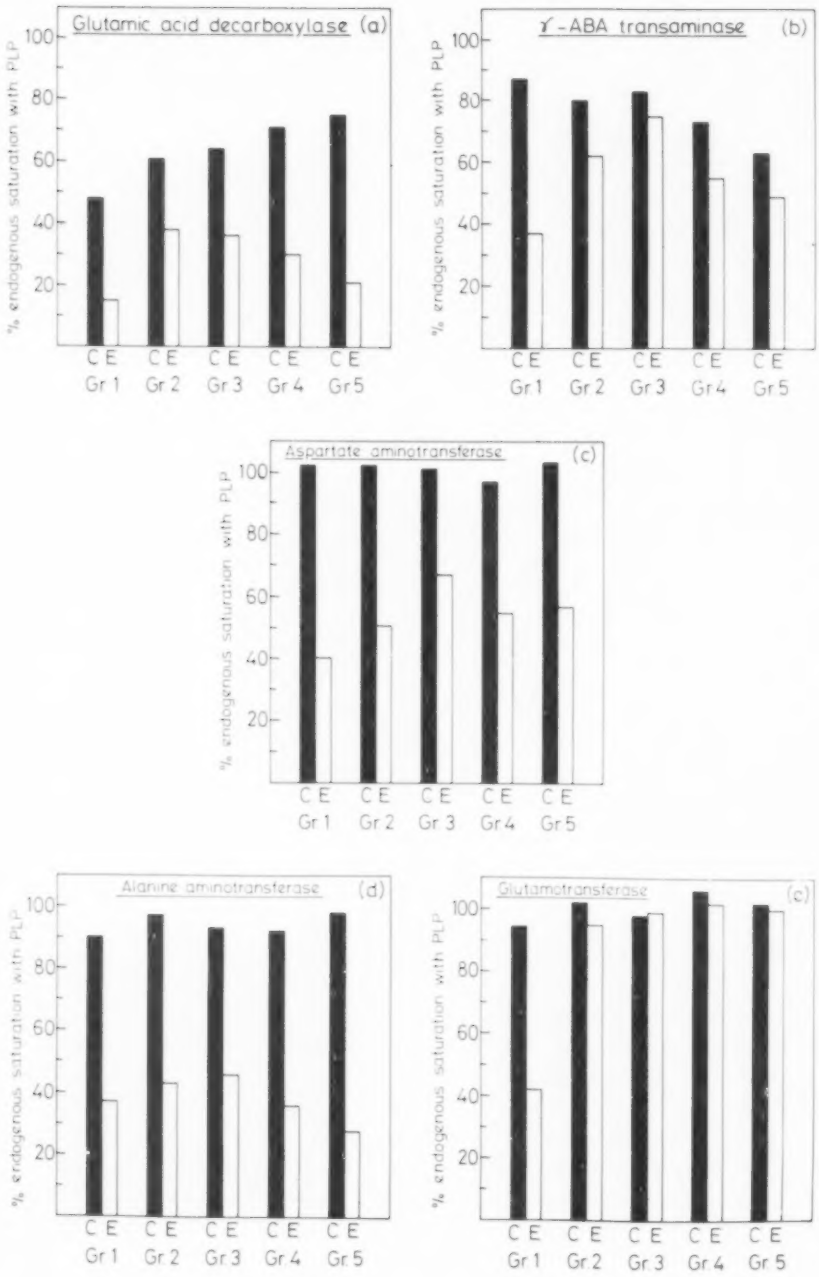

FIG. 2. Per cent endogenous saturation with PLP of brain GAD, GABA-T, AsAT, AlAT and GT as a function of pyridoxine deficiency at different ages in rats.
Gr. - Group C - Control E - Experimental

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the B_6 dependent brain enzymes of the older animals, thereby showing the importance of adequate B_6 requirement for the homeostasis of the aged brain.

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EFFECT OF AGE AND SEX ON THE ASCORBIC ACID CONTENT OF KIDNEY, SKELETAL MUSCLE AND PANCREAS OF COMMON INDIAN TOAD, *BUFO MELANOSTICTUS*

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Abstract – Ascorbic acid decreases from age-group I to II in Kidney, skeletal muscle, and increases in the Pancreas of female. There is no significant change in the Pancreas of male toad. There is an increase in the ascorbic acid content from age group II to III except in the Pancreas of females, where there is a decline of the content. Ascorbic acid remains constant in age-group IV except in the kidney of female, skeletal muscle of both sexes showing an increase and decrease of the value respectively.

Sex difference shows a higher content in kidney of group IV, in skeletal muscle of group II and III and in pancreas of group II and is less in kidney of age-group II of females over their male counterparts.

INTRODUCTION

AGE RELATED studies on ascorbic acid (AA) metabolism (Patnaik, 1971) and its involvement in various functions has been discussed (Padhi, 1980). Ascorbic acid (AA) acts as an anti-aging agent (Panigrahy, 1976), takes part in wound-healing (Bartlett, Jones and Ryan, 1942; Udupa and Singh, 1964), cellular respiration (King, 1950) and declines with age in garden lizard liver (Rao and Patnaik, 1973), skeletal muscle (Haseeb and Patnaik, 1973) and in rat (Kanungo and Patnaik, 1964) has been reported. But effect of age and sex on such an important biochemical component of kidney, the synthesising organ in amphibians (Chatterjee, 1973), skeletal muscle, the tensile strength of which decreases with age and pancreas, the organ which also stores AA (Chinoy, 1977) is largely unknown in the common indian toad. The present work aims at the colorimetric estimation of AA in kidney, skeletal muscle and pancreas of common Indian toad, *Bufo melanostictus*, in relation to age and sex.

MATERIALS AND METHODS

Toads of different sizes were collected from Bhadrak locality during June–November and were grouped into four age-groups taking body-weight and snout to vent (S-V) length into consideration for convenience, for statistical evaluation of data (age-group I, 0–15g; age-group II, 16–31g; age-group III, 32–47g; age-group IV, 48–onwards). That body weight and S-V length increases with age has been reported for garden lizards (Padhi

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and Patnaik, 1978) in captivity. This is the main reason why body weight and S-V length were taken as parameter in aging studies in toads as reported for lizards (Smith, 1964; Eapen and Puspendra, 1972; Tinkle and Selander, 1973; Haseeb and Patnaik, 1973), rat (Devi *et al.*, 1963; Gerlach and Fegeler, 1973). Table 4 shows the range of body-weight, S-V length and the range of tissue weight, taken for AA estimation.

Toads were killed with a blow on their head within 24 hour of their collection. Kidney, pancreas and skeletal muscle (mostly from femoral region) were taken, adherent tissues were cleaned, weighed, homogenised in a low speed all-glass homogenizer (Remi make), and centrifuged in a centrifuge (Remi make) at 2000 rpm for ten minutes. The supernatant was taken for colorimetric estimation of AA following the method of Roe (1967) as modified by Tewary and Pandey (1964).

RESULTS

Table 1 shows the following age-related changes in kidney, skeletal muscle and pancreas of male toad, *Bufo melanostictus*.

In kidney AA show a significant increase with age in absolute value (μg./whole tissue) whereas it indicates a decline from age-group I to II in relative value (mg/100g. wet weight) and remained almost constant thereafter.

In skeletal muscles AA is more in age-groups III and IV over age-groups II and I respectively. There is no significant difference between age-groups I and II and III and IV toads in absolute value. Relative values exhibit a significant decrease from age-group I to II and a rise in the value from age-group II to III which remained almost constant thereafter, without showing any significant change between age-groups IV and I.

Absolute value of pancreas indicate significant increase from age-group I to III and then stabilized in Group IV showing a significant decrease from age-group IV to I. Relative values show more of AA in age-group III over group II but there is no significant difference between age-groups I and II, III and IV and IV and I. Age-related changes of AA in kidney, skeletal muscle and pancreas of female toad (Table 2) are as follows.

Kidney shows a significant rise of AA content with age in absolute value. Relative values indicate a decline of the content from age-group I to II and a steady increase thereafter. On the otherhand, there is a decrease of the content from age-group IV to I.

AA content of skeletal muscle does not show significant difference between age-group I and II but there is a sharp increase from age-group II to III and then a decline from age-group III to IV and IV to I in absolute value. Relative value shows a decline of the content from age-group I to II, III to IV and IV to I but an increase from age-group II to III.

Absolute value of pancreas does not show significant change between age group I and II but an increase from age group II to III, which remained constant thereafter showing a decline from age-group IV to I. Relative value of AA exhibits a sharp increase from age-group I to II and significant decrease from age-group II to III which remained constant thereafter.

Aged-related differences of AA with regard to sex (Table 3) are given below.

Absolute value of kidney shows a higher content in age-groups I and IV females over their male counter-parts. Relative values indicate less in group II and more in group IV females over their male counter-parts. Age group III females show higher content in absolute value while in relative value group II and III females indicate higher content of AA in the skeletal muscle over their male counter-parts.

AA content of pancreas does not indicate any significant difference in absolute value. The relative value show higher content in age-group II females over its male counter-part.

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TABLE 1. EFFECT OF AGE ON THE ASCORBIC ACID CONTENT OF KIDNEY, SKELETAL MUSCLE AND PANCREAS OF MALE TOAD *BUFO MELANOSTICTUS*

Age group	*Kidney*				*Skeletal Muscle*				*Pancreas*			
	a	*'p'*	*b*	*'p'*	*a*	*'p'*	*b*	*'p'*	*a*	*'p'*	*b*	*'p'*
I	2.42 ± 0.22 (7)	<0.001	6.93 ± 0.41 (7)	<0.02	3.19 ± 0.63 (10)	NS	3.91 ± 0.57 (10)	<0.01	0.79 ± 0.13 (10)	<0.02	2.97 ± 0.48 (7)	NS
II	10.92 ± 0.98 (17)	<0.001	5.27 ± 0.47 (17)	NS	2.57 ± 0.39 (14)	<0.01	1.98 ± 0.26 (14)	<0.005	1.38 ± 0.18 (18)	<0.001	1.98 ± 0.15 (17)	<0.02
III	20.10 ± 1.44 (11)	<0.01	5.96 ± 0.48 (11)	NS	5.12 ± 0.74 (8)	NS	3.53 ± 0.35 (8)	NS	2.90 ± 0.34 (9)	NS	3.15 ± 0.52 (8)	NS
IV	26.11 ± 1.22 (10)	<0.001	6.15 ± 0.29 (13)	NS	5.55 ± 0.74 (8)	<0.05	4.03 ± 0.44 (8)	NS	2.45 ± 0.41 (11)	<0.001	2.01 ± 0.28 (11)	NS

(a = μg ascorbic acid/whole tissue; b = mg ascorbic acid/100 G. net weight. Number in parantheses indicate animals used. Values are average ± SEM. Comparison 'p' between age group I & II, II & III, III & IV and IV & I).

TABLE 2. EFFECT OF AGE ON THE ASCORBIC ACID CONTENT OF KIDNEY, SKELETAL MUSCLE AND PANCREAS OF FEMALE TOAD

Age group	*Kidney*				*Skeletal Muscle*				*Pancreas*			
	a	*'p'*	*b*	*'p'*	*a*	*'p'*	*b*	*'p'*	*a*	*'p'*	*b*	*'p'*
I	6.51 ± 0.79 (11)	<0.025	5.63 ± 0.51 (11)	<0.01	2.94 ± 0.25 (8)	NS	6.52 ± 1.52 (6)	<0.05	0.71 ± 0.28 (6)	NS	1.71 ± 0.57 (6)	<0.005
II	9.58 ± 1.00 (16)	<0.001	3.66 ± 0.40 (16)	<0.005	2.88 ± 0.26 (10)	<0.001	2.91 ± 0.19 (10)	<0.005	1.09 ± 0.19 (12)	<0.001	4.24 ± 0.43 (9)	<0.01
III	22.93 ± 1.54 (10)	<0.001	5.31 ± 0.33 (11)	<0.001	8.22 ± 0.89 (11)	<0.005	5.27 ± 0.61 (12)	<0.025	2.92 ± 0.32 (12)	NS	2.61 ± 0.26 (10)	NS
IV	49.37 ± 4.69 (9)	<0.001	7.26 ± 0.41 (8)	<0.001	4.36 ± 0.40 (8)	<0.02	3.37 ± 0.48 (9)	NS	2.85 ± 0.53 (8)	<0.005	1.86 ± 0.25 (7)	NS

(a = μg ascorbic acid/whole tissue; b = MG ascorbic acid,/100 G. wet weight. Number in parantheses indicate animals used. Values are average ± SEM. Comparison 'p' between age groups I & II, II & III, III & IV and IV & I).

TABLE 3. EFFECT OF SEX ON THE ASCORBIC ACID CONTENT OF KIDNEY, SKELETAL MUSCLE AND PANCREAS OF COMMON INDIAN TOAD

Age group/ Sex	*Kidney*				*Skeletal Muscle*				*Pancreas*			
	a	*'p'*	*b*	*'p'*	*a*	*'p'*	*b*	*'p'*	*a*	*'p'*	*b*	*'p'*
I												
Male	2.42 ± 0.22 (7)		6.93 ± 0.41 (7)		3.19 ± 0.63 (10)		3.91 ± 0.57 (10)		0.79 ± 0.13 (10)		2.97 ± 0.48 (7)	
		< 0.001		NS		NS		NS		NS		NS
Female	6.51 ± 0.79 (11)		5.63 ± 0.51 (11)		2.94 ± 0.25 (8)		6.52 ± 1.52 (6)		0.71 ± 0.28 (6)		1.71 ± 0.57 (6)	
II												
Male	10.92 ± 0.98 (17)		5.27 ± 0.47 (17)		2.57 ± 0.39 (14)		1.98 ± 0.26 (14)		1.38 ± 0.18 (18)		1.98 ± 0.15 (17)	
		NS		< 0.02		NS		< 0.02		NS		< 0.001
Female	9.58 ± 1.00 (16)		3.66 ± 0.40 (16)		2.88 ± 0.26 (10)		2.91 ± 0.19 (10)		1.09 ± 0.19 (12)		4.24 ± 0.43 (9)	
III												
Male	20.10 ± 1.44 (11)		5.96 ± 0.48 (11)		5.12 ± 0.74 (8)		3.53 ± 0.35 (8)		2.90 ± 0.34 (9)		3.15 ± 0.52 (8)	
		NS		NS		< 0.02		< 0.025		NS		NS
Female	22.93 ± 1.54 (10)		5.31 ± 0.33 (11)		8.22 ± 0.89 (11)		5.27 ± 0.61 (12)		2.92 ± 0.32 (12)		2.61 ± 0.26 (10)	
IV												
Male	26.11 ± 1.22 (10)		6.15 ± 0.29 (13)		5.55 ± 0.74 (8)		4.03 ± 0.44 (8)		2.45 ± 0.41 (11)		2.01 ± 0.28 (11)	
		< 0.001		< 0.05		NS		NS		NS		NS
Female	49.37 ± 4.69 (9)		7.26 ± 0.41 (8)		4.36 ± 0.40 (8)		3.37 ± 0.48 (9)		2.85 ± 0.53 (8)		1.86 ± 0.25 (7)	

(a = μg ascorbic acid/whole tissue, b = mg ascorbic acid/100 G. wet weight, number in parantheses indicate animals used, values are average ± SEM. Comparison 'p' between age groups I & II, II & III, III & IV and IV & I)

TABLE 4. PARTICULARS OF ANIMALS AND TISSUES USED

Age group	*Sex*	*Range of body weight (g)*	*Range of S-V length (cm)*	*Range of kidney weight (mg)*	*Range of skeletal muscle (mg)*	*Range of pancreas weight (mg)*
I	Male	3 – 15	3.0 – 6.0	43 – 143	32 – 154	10 – 37
	Female	3 – 15	2.8 – 6.2	67 – 192	27 – 167	26 – 56
II	Male	17 – 30	5.8 – 7.7	127 – 318	98 – 196	29 – 154
	Female	16 – 34	6.0 – 7.7	174 – 405	88 – 162	27 – 154
III	Male	35 – 44	7.5 – 8.5	226 – 400	88 – 173	36 – 83
	Female	36 – 47	7.3 – 9.1	238 – 510	103 – 173	43 – 176
IV	Male	47 – 81	8.0 – 10.0	310 – 559	100 – 191	66 – 257
	Female	47 – 110	8.0 – 11.5	410 – 803	106 – 170	110 – 281

DISCUSSION

Our findings that there is a fall of AA level from age-group I to II initially which rises in age-group III and stabilised thereafter in kidney and skeletal muscle of male (Table 1), whereas an increase in age-group IV in the kidney of female toad (Table 2) are in conformity with the age-related changes of AA in the garden lizard brain (Padhi and Patnaik, 1975).

The results on pancreas of the male toad (Table 1) does not signify any change between age-group I and II but a high content in age group III which remained constant thereafter. Our observations on pancreas coincides with the age-related changes of glycogen content in the skeletal muscle of Calotes versicolor (Haseeb and Patnaik, 1973).

Skeletal muscle of the female toad (Table 2) show significant decrease of AA from age group I to II and III to IV as observed for age-related changes of DNA content in the skin of male garden lizard (Mishra and Patnaik, 1974).

Age related changes of pancreas of the female toad (Table 2) show an increase in AA content from age-group I to II and then a decrease from age group II to III which remained constant thereafter in age group IV are identical with the age-related changes of Ascorbic Acid content in the kidney of male garden lizard (Padhi, 1980).

Our observations on sex difference show less content in age-group II kidney of males, whereas high content in age-groups IV, II and III and II in the kidney, skeletal muscle and pancreas of females over their male counter-parts suggest differential utilisation of AA in various tissues. This is in contrast to the observation as reported for serum calcium (Patnaik and Singh, 1964) where there is no effect of sex. But Padhi (1982) observed higher AA content in the kidney of male garden lizard, *Calotes versicolor* and in the kidney of female lizard, *Hemidactylus flaviviridis.*

In brief, it can be said from the above that the age-related changes in poikilo-thermic vertebrates so far studied follow almost similar trend with some minor deviations.

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EFFECT OF AGE, STARVATION AND CIRCADIAN RHYTHM ON THE ASCORBIC ACID CONTENT AND SUCCINIC DEHYDROGENASE ACTIVITY OF THE KIDNEY OF MALE GARDEN LIZARD, *CALOTES VERSICOLOR*

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Abstract – Both ascorbic acid content and SDH activity of kidney reached peak levels during sexual maturity and then declined. Starvation stress (21 days) induced low levels of ascorbic acid content and SDH activity of the kidney. Nocturnal levels of ascorbic acid content and SDH activity were significantly lower than the diurnal values.

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INTRODUCTION

SINCE A NUMBER of physiological factors are known to influence the status of ascorbic acid through their action on its synthesis and oxidation (Padhi, 1980), it was considered of interest to assess the level of ascorbic acid in the kidney of garden lizards under various experimental conditions. Since ascorbic acid is known to stimulate the activity of the mitochondrial marker enzyme, succinic dehydrogenase, SDH (Moon *et al.*, 1976) studies were also extended to the estimation of enzyme activity. This, it was thought, would add credence to the fact that the factors influencing ascorbic acid metabolism may in turn effect the respiratory metaboism of garden lizards.

The physiological factors chosen were age, starvation and circadian rhythm. In general, none of these factors have received due consideration in reptilian metabolism (Bennett and Dawson, 1976).

MATERIALS AND METHODS

Experimental design

Age-related studies: Male garden lizards of four different age-groups as mentioned below were collected from nature during October-November (for ascorbic acid) and November-December (for SDH) and were maintained in the laboratory as described (Padhy and Patnaik, 1979).

Age group	Range of body wt. (g)	Range of S-V length (cm)	Approximate age (years).
I	5–7	5.5– 8.8	Under 1 year.
II	18–30	8.1–10.2	Under 1 year
III	31–45	9.7–10.6	1 year.
IV	46–64	10.7–12.0	2 to 3 years.

Starvation:—Male garden lizards having a body weight range 18–45 g and S-V length 8.8–10.9 cm. collected during April–May (for ascorbic acid and May–June (for SDH) were used. The animals were categorized as initial control, fed control and starved (14 and 21 days) groups. Lizards grouped as "initial control" were sacrificed on the day of the start of the experiment. The "fed control" group of lizards were fed with goat liver on alternate day and the starved group of lizards were provided with tap water only.

Circadian rhythm: Male garden lizards having a body weight range 16–31 g and S-V length range 8.3–10.1 Cm collected during December–January were maintained and killed during different hours (1 AM, 7 AM, 1 PM and 7 PM) to ascertain the circadian rhythm in ascorbic acid content and SDH activity of the kidney.

Ascorbic acid estimation: Total free ascorbic acid was extracted twice from the kidney using 5 ml and 2.5 ml of 5% T CA respectively. The extract was used for ascorbic acid estimation following the method of Roe (1967) as modified by Tewary and Pandey (1964).

SDH activity:—The method of studying SDH activity was essentially the same as described by Kun and Abood (1949). A tissue homogenate (100 mg./2.5 ml. of 0.25 M sucrose) was prepared in a Scientronic (New Delhi) porter Elvehjem type of homogenizer. The reaction mixture for enzyme activity consisted of 1 ml of 4% tissue homogenate, 0.5 ml. of 0.1 M sodium succinate in M/15 Phosphate buffer, PH 7.4 and 0.5 ml of triphenyl tetrazolium chloride (0.3%). A blank without the substrate (sodium succinate) was also run simultaneously. The formazan produced was extracted in acetone measured at 505 mμ in spekol (Carlzeiss).

RESULTS

Age-changes in ascorbic acid content and SDH activity

The relative ascorbic acid level showed a rise during maturity and then declined. On the other hand the absolute value reached the peak level at full maturity followed by a decline thereafter (Table 1).

TABLE 1. EFFECT OF AGE ON THE ASCORBIC ACID (AA) CONTENT AND SDH ACTIVITY OF THE KIDNEY OF MALE GARDEN LIZARD

Age-group	*mg.AA/100g. wet wt. Average ± SEM (No. of animals)*	'p'	*μg.AA/Whole kidney Average ± SEM (No. of animals)*	'p'	*SDH activity O.D./mg. protein/hr. Average ± SEM (No. of animals)*	'p'
I	10.76 ± 0.94 (8)		5.86 ± 0.95 (8)		0.083 ± 0.004 (8)	
		NS		<0.001		NS
II	12.39 ± 0.89 (7)		12.42 ± 1.14 (7)		0.101 ± 0.012 (7)	
		<0.05		<0.01		NS
III	9.38 ± 0.80 (6)		19.54 ± 2.11 (6)		0.097 ± 0.005 (6)	
		NS		<0.1		NS
IV	7.58 ± 0.91 (7)		14.53 ± 2.18 (7)		0.082 ± 0.007 (7)	

TABLE 2. EFFECT OF STARVATION ON THE ASCORBIC ACID (AA) CONTENT AND SDH ACTIVITY OF THE KIDNEY OF MALE GARDEN LIZARD

Condition	*mg.AA/100g. wet wt. Average ± SEM (No. of animals)*	*'p'*	*μg.AA/Whole kidney Average ± SEM (No. of animals)*	*'p'*	*SDH activity O.D./mg. protein/hr. Average ± SEM (No. of animals)*	*'p'*
Initial control	12.04 ± 1.81 (10)		14.59 ± 1.86 (10)		0.080 ± 0.003 (6)	
Fed control	4.58 ± 0.58 (8)	<05	7.53 ± 1.56 (8)	<0.01	0.044 ± 0.003 (7)	<0.01
14 days starved	6.11 ± 0.89 (7)	NS	6.75 ± 0.97 (7)	NS	0.053 ± 0.006 (6)	NS
Fed Control	5.58 ± 0.81 (10)	<0.001	7.17 ± 0.96 (10)	<0.001	0.081 ± 0.011 (9)	<0.01
21 days starved	3.47 ± 0.32 (11)	<0.05	3.49 ± 0.44 (11)	<0.001	0.050 ± 0.005 (8)	<0.05

The SDH activity of kidney showed the same age-related pattern as observed for relative ascorbic acid level (Table 1).

Effect of starvation

There were significant differences between both the parameters (ascorbic acid and SDH activity) of kidney of fed control garden lizards and initial controls. A period of 14 days starvation did not influence the ascorbic acid content and SDH activity of the content but a longer duration of starvation resulted in a significant decline (Table 2).

Circadian rhythm

The ascorbic acid content of the kidney was lowest at 1:00 a.m. and then reached a peak level at 1:00 p.m. remaining constant thereafter till 7:00 p.m. (Table 3).

On the other hand, the SDH activity was lowest at 7:00 p.m. reached a peak level at 7:00 a.m., and then continued to decline (Table 3).

TABLE 3. EFFECT OF CIRCADIAN RHYTHM ON THE ASCORBIC ACID (AA) CONTENT AND SDH ACTIVITY OF KIDNEY OF MALE GARDEN LIZARD

Hour	*mg.AA/100g. wet wt. Average ± SEM (No. of animals)*	*'p'*	*μg.AA/Whole kidney Average ± SEM (No. of animals)*	*'p'*	*SDH activity O.D./mg. protein/hr. Average ± SEM (No. of animals)*	*'p'*
7 A.M.	13.04 ± 2.30 (10)		13.44 ± 2.60 (10)		0.089 ± 0.008 (6)	
		NS		NS		<0.05
1 P.M.	15.41 ± 2.40 (9)		13.63 ± 1.95 (9)		0.061 ± 0.002 (5)	
		NS		NS		<0.01
7 P.M.	14.90 ± 2.10 (8)		18.70 ± 2.97 (8)		0.049 ± 0.001 (6)	
		<0.05		<0.05		<0.01
1 A.M.	9.42 ± 1.12 (9)		10.92 ± 1.41 (9)		0.066 ± 0.004 (7)	

DISCUSSION

That the ascorbic acid metabolism declines with age in mammals has been discussed (Patnaik, 1971, a). In garden lizard liver, skeletal muscle and skin the ascorbic acid content declined with age but in brain it remained almost constant (for review Patnaik, 1980). Such age-related changes were thought due to changes in rate of synthesis in the kidney and in the rate of its utilization in the respective tissues. Considering the fact that there is negligible oxidation of ascorbic acid in the kidney (Padhi, 1980), the ascorbic acid content of kidney may be taken to represent a crude measure of ascorbic acid synthesis. It seems that the synthesis reaches the peak level during sexual maturity and declines thereafter.

Wilson (1973) observed that the mitochondrial respiratory enzyme, SDH reached a peak level of activity in liver and kidney of mice at 18 months and then declined. Our results on lizard kidney almost follow the same pattern. Thus, it seems that the respiratory metabolism of kidney declines in cold animals due mainly to a decreased activity of the enzymes. Basically, therefore, a common pattern of aging prevails at cellular level. Since the pattern of age-changes in ascorbic acid content and SDH activity are similar one might suggest that such changes are inter-related.

The ability to survive prolonged fast is a characteristic feature of reptiles. A number of bio-chemical components of the blood of rattle snakes (Martin and Baby, 1973), lipid content of nervous tissues of monitor lizards (Khatil and Abdel-Messeiah, 1959) and glycogen content of various tissues of turtles (Penney and Papademos, 1975) remain unchanged even after several days of starvation. On the other hand, Gist (1972) reported a decline in the glycogen reserves of liver and muscle of *Anolis carolinensis*. Thus it seems that conflicting reports exist on the biochemical alterations in the tissues of starving reptiles.

Moderate days (14 days) of starvation did not influence the ascorbic acid content and SDH activity of the kidney of garden lizards. But following 21 days of starvation both the biochemical constituents declined. Earlier Sinha and Kanungo (1967) observed a decline in the ascorbic acid content of liver and muscle of scorpions during late days of starvation. Stripe and Comporti (1965) reported a decline in the synthesis of ascorbic acid in rat liver following starvation stress. Our findings that the ascorbic acid content of kidney declines after a starvation period of 21 days, suggests that the rate of synthesis is deranged during this period. Since the activity of the mitochondrial enzyme SDH declines during the period of starvation, the respiratory metabolism of kidney seems to be reduced appreciably. The fact that the ascorbic acid content and the SDH activity follow a similar pattern of change during starvation again suggests a correlation between them.

Several workers (Heath, 1962; Evans 1966; Saint Girons, 1971) have reported circadian patterns in the activity of reptiles. However, studies on the correlations between these patterns and metabolism have been a subject of limited investigation. Kramer (1934) reported a low metabolic rate in *Lacerta* at night. Similarly Roberts (1968) also observed the nocturnal metabolic rate to be about ⅓ of diurnal. The oxygen consumption of *Sceloporus cyanogenys* (Songdahl and Hutchison, 1972) was higher during light phase than during the nocturnal phase of the daily cycle. In *Xeutusia henshawi* the nocturnal metabolism was as little as 30% of the diurnal value.

That nocturnal level of ascorbic acid content and SDH activity of kidney were lower than diurnal values suggest that circadian rhythm of metabolism in reptiles may allow significant conservation of energy during the inactive phase of the daily cycle (Bennett and Dawson, 1976).

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AGE-RELATED CHANGES IN THE WATER AND ELECTROLYTE METABOLISM IN MALE RATS OF THE WISTAR/TW STRAIN

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Abstract—The changes of water intake, urinary volume, ratio of the water intake to the urinary output (water turnover rate) and urinary concentrations of sodium, potassium and chloride ions were investigated in male rats of the inbred Wistar/Tw strain during aging. Marked increase of the water intake, the urinary output and the water turnover rate as well as marked decrease of the urinary electrolyte concentrations were observed at the age of over 16 months. First signs of increase in the water intake and the water turnover rate were observed at the age of 12–13 months, but the initial reduction of urinary electrolyte concentrations was detected as early as at 7–9 months of age.

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INTRODUCTION

WE HAVE reported the occurrence of polydipsia and polyuria, with the decrease of urinary electrolyte concentrations, in male rats of the inbred Wistar/Tw strain at the age of 17 months (Kobayashi and Kawashima, 1980). Water intake per 100 g body weight in aged rats was about 1.7 times that in 5-month-old rats and urinary volume was about 3.6 times. According to Everitt (1958) and Friedman, Friedman and Nakashima (1967), significant increase in the water intake and the urine production per metabolic body size or body weight has not been observed in their Wistar strain males until at the age of 24 months. A slight but statistically significant increase in the water intake and the urinary output, to 1.1 and 1.3 times respectively, was noted only at about 32 months of age (Everitt, 1958). Earlier and clearer increase of the water intake and the urinary volume in our male Wistar/Tw rats than in the same strain maintained in other laboratories encourages us to use our substrain as a research model of aging in the water metabolism.

The purpose of the present study is to clarify at what age the water intake and the urinary volume begin to increase, and how the concentrations of urinary sodium, potassium and chloride ions change during the course of aging.

MATERIALS AND METHODS

Male rats of the inbred Wistar/Tw strain of various ages were used in the present experiments. They were fed laboratory chow (CA-1, CLEA Japan Inc.) and water *ad libitum*. The amounts of food, water intake and

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urinary volume were measured in each rat kept in an aluminum metabolic cage in which the urine could be collected without contamination with feces. The concentrations of urinary sodium and potassium ions were determined by an atomic absorption spectrophotometer (Hitachi 180-50), while that of chloride ion by a Bucher digital chloridometer. Total daily excretion of these ions was calculated by the urinary concentrations of these ions and daily urinary volume. The statistical comparisons between two groups were performed by Student's *t*-test.

RESULTS

Body weight and food consumption

The body weight increased until about 9 months of age and then it kept constant until 19 months, with some fluctuations (Figure 1). By contrast, the daily food intake per 100 g body weight decreased until about 9 months of age followed by a steady level until 19 months (Figure 1).

Water intake and urinary output

Figure 2a shows the changes in daily water intake and urinary output per 100 g body weight during aging. There was a decrease in the water intake from the age of 3 months to the age of 9 months. The urinary output, however, did not show any significant change until the age of 9 months. The water intake of the 12 to 13-month-old rats was significantly greater than that of 7 to 9-month-old animals, but not significantly different from that of 3 to 4-month-old animals. By contrast, slight increase of the urinary output was observed in the rats at the age of 12 to 13 months as compared with that in the animals at the age of 3 to 4 months. Marked increases of both the water intake and the urinary output were detected after the animals reached the age of 16 months. In the rats at the age of over 16 months, the water intake was 1.8 times as much as that at 3 to 4 months, while the urine production was 5.7 times.

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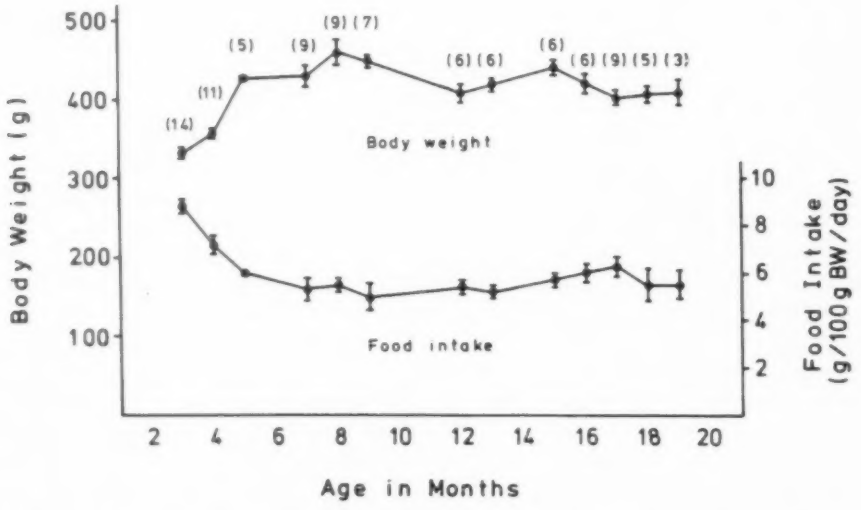

FIG. 1. Changes in body weight and food intake in male rats of the Wistar/Tw strain during aging. Vertical bars designate standard errors of means. Numbers of rats are given in parentheses.

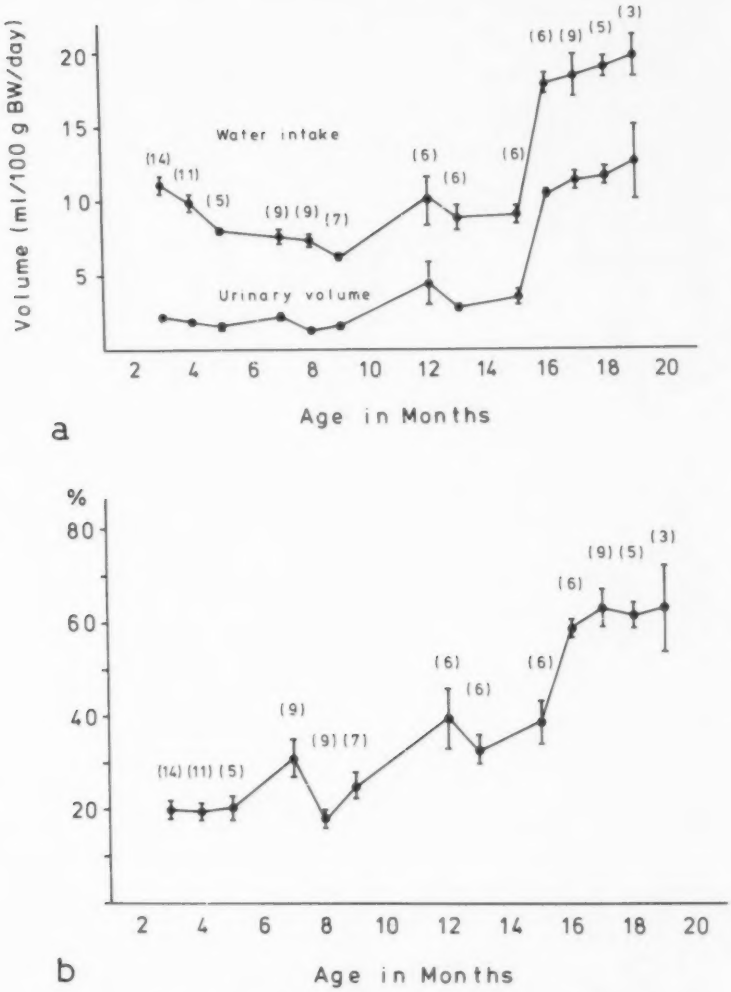

FIG. 2. Changes in water metabolism during aging in male rats of the Wistar/Tw strain. Vertical bars indicate standard errors of means. Numbers of rats are given in parentheses.

FIG. 2a. Changes in water intake and urinary volume. The statistical analyses of water intake: 3–4 months ($n = 25$) vs 7–9 months ($n = 25$), $p < 0.001$; 7–9 months vs 12–13 months ($n = 12$), $p < 0.02$; 12–13 months vs 16–19 months ($n = 23$), $p < 0.001$; 3–4 months vs 12–13 months, n.s. That of urine production: 3–4 months vs 7–9 months, n.s.; 7–9 months vs 12–13 months, $p < 0.05$; 12–13 months vs 16–19 months, $p < 0.001$; 3–4 months vs 12–13 months, $p < 0.05$.

FIG. 2b. Changes in water turnover rate (ratio of urinary volume to water intake). The statistical analyses of changes: 3–4 months vs 7–9 months, n.s.; 7–9 months vs 12–13 months, $p < 0.01$; 12–13 months vs 16–19 months, $p < 0.001$; 3–4 months vs 12–13 months, $p < 0.005$.

The ratio of the urinary volume to the water intake (water turnover rate) increased slightly at the age of 12 to 13 months as compared with that at the age of 3 to 4 or 7 to 9 months (Figure 2b). The water turnover rate of the rats at the age of 7 to 9 months was not significantly different from that at the age of 3 to 4 months, notwithstanding the water in-

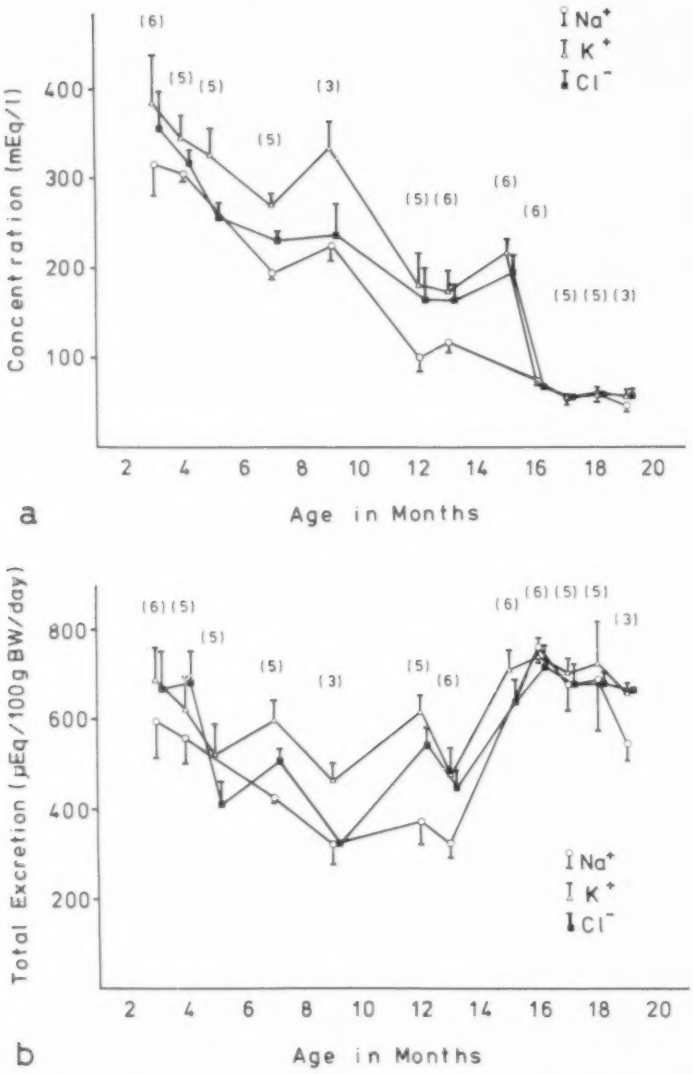

FIG. 3. Changes in electrolyte metabolism in male rats of the Wistar/Tw strain. Vertical bars indicate standard errors of means. Numbers of rats are given in parentheses.

FIG. 3a. Changes in urinary concentrations of electrolytes. The statistical analyses of changes: Sodium, 3–4 months ($n = 11$) vs 7–9 months ($n = 8$), $p < 0.001$; 7–9 months vs 12–13 months ($n = 11$), $p < 0.001$; 12–13 months vs 16–19 months ($n = 19$), $p < 0.001$. Potassium, 3–4 months vs 7–9 months, n.s.; 7–9 months vs 12–13 months, $p < 0.002$; 12–13 months vs 16–19 months, $p < 0.001$; 3–4 months vs 12–13 months, $p < 0.001$. Chloride, 3–4 months vs 7–9 months, $p < 0.005$; 7–9 months vs 12–13 months, $p < 0.01$; 12–13 months vs 16–19 months, $p < 0.001$.

FIG. 3b. Changes in total daily excretion of urinary electrolytes. The statistical analyses of changes: Sodium, 3–4 months vs 7–9 months, $p < 0.01$; 7–9 months vs 12–13 months, n.s.; 12–13 months vs 16–19 months, $p < 0.001$; 3–4 months vs 16–19 months, n.s. Potassium, 3–4 months vs 7–9 months, n.s.; 7–9 months vs 12–13 months, n.s.; 12–13 months vs 16–19 months, $p < 0.002$; 3–4 months vs 16–19 months, n.s. Chloride, 3–4 months vs 7–9 months, $p < 0.02$; 7–9 months vs 12–13 months, n.s.; 12–13 months vs 16–19 months, $p < 0.01$; 3–4 months vs 16–19 months, n.s.

take of the former rats was considerably decreased. The water turnover rate was decidedly higher at the age of 16 months, which indicates a preferential increase in the urinary output after this age.

Urinary electrolytes

Changes in the concentrations of urinary sodium, potassium and chloride ions during aging are shown in Figure 3a. The significant decrease of the urinary concentrations of sodium and chloride ions was observed in the rats at the age of 7 to 9 months as compared with those at 3 to 5 months, although the increase of urine production was not observed at 7 to 9 months of age. Further decrease of the urinary concentrations of sodium, potassium and chloride ions was found in rats at the age of 12 to 13 months and over 16 months compared with younger animals.

Total daily excretion of sodium and chloride ions decreased significantly until the age of 9 months, and that of potassium ion showed a similar tendency (Figure 3b). Total excretion of all three ions at 12 to 13 months of age was not significantly different from that at 7 to 9 months, but at the age more than 16 months, it increased to the level of 3 to 4-month-old animals.

DISCUSSION

The present results are consistent with our previous findings (Kobayashi and Kawashima, 1980; Kawashima and Kobayashi, 1982) that polydipsia and polyuria, with the reduction of urinary concentrations of sodium, potassium, magnesium and chloride ions, and glucose, took place in the middle-aged male rats of the Wistar/Tw strain. The significant increase in water turnover rate and the reduction in urinary electrolyte concentrations at the age of 12 to 13 months in Wistar/Tw strain suggest that the mode of water and electrolyte metabolism begins to change earlier than the apparent occurrence of polydipsia and polyuria. Furthermore, the reduction of the urinary electrolyte concentrations at the age of 7 to 9 months suggests that the initial change in electrolyte metabolism may be traced as early as at this age in our Wistar/Tw males, although there is a possibility that the reduction of the urinary electrolyte concentrations at 7 to 9 months of age compared with that at 3 to 4 months is a physiological maturational change and can not be considered to be the initial sign of polydipsia.

In contrast to the present results in Wistar/Tw rats, male rats of the inbred Sprague-Dawley/Tw strain similarly maintained in this laboratory showed a decrease in the water intake but no change in the urinary production per 100 g body weight at the age of 17 months (Kawashima and Kobayashi, 1982). Thus, the induction of polydipsia and polyuria in aged rats of the Wistar/Tw strain was not due to the condition of maintenance but to the strain difference. Similar to the results in Sprague-Dawley/Tw rats, Friedman, Friedman and Nakashima (1967) reported that in 24-month-old rats of the Wistar strain, the water intake per body weight was smaller than that in 4-month-old animals, but that the urinary production was approximately the same as that in the younger rats. Everitt (1958) reported a decrease in the daily water intake per metabolic body size of the Wistar rats at the age of 17 months compared with that at 8 months, but he found a senile change on the water metabolism in rats at about 32 months of age, the water intake being 1.1 times and the urinary production being 1.3 times as much as those in rats at about 8 months of age. Therefore, the age-related changes observed in the water intake and the urinary output

seem to appear earlier and more clearly in our Wistar/Tw rats than that in the animals of the same strain kept in other laboratories or in our Sprague-Dawley/Tw rats.

The water turnover rate was not increased in Wistar/Tw female rats at the age of 16 months, but it increased at 24 months (Kobayashi and Kawashima, 1982). Males of the Sprague-Dawley/Tw strain in this laboratory also showed an increase in the water turnover rate at 17 months of age as compared with that at 5 months of age (Kawashima and Kobayashi, 1982). Friedman, Friedman and Nakashima (1967) and Turkington and Everitt (1976) also observed the increase in the water turnover rate at the age of about 24 months in male rats of the Wistar strain. Accordingly, the increase in the water turnover rate seems to be a common parameter of aged rats, although significant changes appear earlier in our Wistar/Tw males.

Gradual decrease of the urinary electrolyte concentrations during aging are consistent with our previous findings (Kobayashi and Kawashima, 1980). Friedman, Friedman and Nakashima (1967) reported the reduction in the urinary concentrations of sodium ion to about 74 percent (calculated from their data) and potassium ion to about 72% in the Wistar male rats at the age of 17–24 months in comparison with those at 4–7 months. Therefore, it is apparent that the changes in the urinary electrolyte concentrations observed in the aged males of the Wistar/Tw strain were more pronounced than in the animals used by Friedman, Friedman and Nakashima (1967).

Rodeck, Lederis and Heller (1960) reported that the amounts of chrome-alum-hematoxyline positive neurosecretory material and the content of antidiuretic hormone (ADH) in the neural lobe of 12 to 24-month-old rats were not significantly different from those of 3 to 6-month-old rats, although a decrease of neurosecretory material was observed in rats over 24 months of age. Similar results have been reported by Turkington and Everitt (1976) that there was no difference in the plasma ADH concentrations between 7-month-old and 13-month-old rats, but the levels in the two groups of old rats (23 and 30 months) were significantly lower than those in younger rats (7 and 13 months). Dicker and Nunn (1958) have reported that injection of hypertonic salt solution or ADH administration to rats at the age of 12 months produced smaller antidiuretic effect compared with that in 3-month-old animals. They also noted that the urine excretion after water deprivation was greater in old rats. Thus, it is possible that the changes in the water and electrolyte metabolism do not occur before about 12 months in rats of these authors, however, the responsiveness to salt load or to ADH is diminished before 12 months.

In Wistar/Tw males at the age of 7 to 9 months, morphological degeneration and the increase in volume of the kidney, an increase in relative volume of the neural lobe, a slight decrease in the amount of neurosecretory material in the neural lobe and an increase in plasma ADH concentration were found compared with those at the age of 3 months (Kobayashi, 1981; Kawashima and Kobayashi, 1982). These findings may support the view that water and electrolyte metabolism begins to change at the age of 7 to 9 months, which causes the eventual development of polydipsia and polyuria at 16 months of age in Wistar/Tw strain males.

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AGE INFLUENCE ON D-GLUCOSE ABSORPTION DEPENDING ON SODIUM CONCENTRATION IN THE LUMEN OF RAT SMALL INTESTINE

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Abstract—The influence of decreased sodium concentration on D-Glucose jejunal and ileal absorption has been investigated in rats of three groups of different ages: 21–23 days, 2–3 months and 18 months old, using a perfusion system *in vivo*.

In all the cases studied there was a decrease of D-Glucose absorption with reduced sodium concentrations.

The ileum of 21–23 days old animals showed the greatest inhibition of D-Glucose absorption when sodium was substituted partially or totally by Tris-HCl.

INTRODUCTION

SINCE CRANE in 1960 postulated "the sodium gradient hypothesis" a number of studies have been made to support it. The sodium dependence of intestinal sugar transport has been confirmed *in vitro* (Crane, R.K. 1977; Schultz, S.G. 1977) and *in vivo* (Debnam, E.S. 1979; Ortiz, M. *et al.*, 1979) in rats, but other results show only a small influence of sodium concentration (Forster, H. 1973).

Because of differences observed in intestinal absorption depending on the animals' age (Scharrer, E. *et al.*, 1979; Younoszai, M.K., and Lynch, A. 1975; Younoszai, M.K. 1979) we have investigated whether D-Glucose absorption could be affected to the same extent by decreased sodium concentrations in the medium, at different stages of life.

To define the functional role of sodium on D-Glucose jejunal and ileal absorption with maturation, we have used an *in vivo* perfusion technique in rats of different ages.

MATERIALS AND METHODS

The experiments were carried out on Wistar rats of both sexes divided into three groups of different ages: 21–23 days, 2–3 months and 18 months old, after 18 hours fasting with water *ad libitum*.

Urethane (12.5%) intraperitoneally at 10 ml/Kg was used to anesthetized the animals and a laparotomy was performed isolating a section of jejunum and ileum between two glass cannulae connected to a perfusion system equipped with a peristaltic pump (Ponz, F. *et al.*, 1979). The solution was perfused with recirculation at a rate of 3.5 ml/min for 20 minutes periods and aliquots were taken at 1, 5, 10 and 20 minutes.

The fluid temperature entering the intestine was adjusted to 37 °C by intercalating a coil immersed in a ther-

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moregulated bath in the circuit to prevent the influence of temperature variation on intestinal absorption of glucose (Rodriguez, M.J. *et al.*, 1982).

The solutions consisted on D-Glucose 10 mM added to NaCl solution or Tris-HCl in various proportions to obtain sodium concentrations of 154, 77, 38.5 and 0 meq/l and to preserve the osmolarity.

The perfusion system was washed after each period with a solution chemically identical to that used in the subsequent period minus the sugar.

The glucose concentration in the perfused solution was determined after perfusion by a chemical method (Nelson, N. 1944; Somogyi, M. 1945) and the glucose absorbed was calculated by substracting the final glucose concentration from the initial one and expressed as μmols absorbed per cm^2 of the intestinal segment perfused, determined at the end of the experiment.

The *t*-Student test was used for the statistical studies.

RESULTS

We found that the amount of D-Glucose absorbed was greater in jejunum than in ileum in the three groups studied, showing the youngest animals to have the greatest absorption in the two intestinal segments (jejunum and ileum) (Tables 1 and 2).

TABLE 1. D-GLUCOSE JEJUNAL ABSORPTION IN RATS OF DIFFERENT AGES ON DEPENDING SODIUM CONCENTRATION AT ONE TO TWENTY MINUTE PERIODS. EACH DATUM IS THE MEAN ± S.E.M. THE NUMBER OF ANIMALS IS IN PARENTHESIS.

	D-Glucose μmols/cm²				
Time (min.)	*Na^+ meq/l: 154*	*77*	*38.5*	*0*	*Age*
1	2.47 ± 0.34 (3)	2.18 ± 0.63 (4)	1.76 ± 0.56 (4)	1.32 ± 0.31 (3)	*21–23 days*
5	3.00 ± 0.34 (4)	2.89 ± 0.31 (4)	2.13 ± 0.40 (6)	1.72 ± 0.42 (4)	
10	3.99 ± 0.32 (4)	3.91 ± 0.46 (4)	2.67 ± 0.24 (3)	2.25 ± 0.44 (4)	
20	4.81 ± 0.49 (5)	4.77 ± 0.44 (4)	3.88 ± 0.19 (4)	3.40 ± 0.62 (3)	
1	1.08 ± 0.15 (7)	0.95 ± 0.12 (7)	0.65 ± 0.30 (4)	0.53 ± 0.21 (5)	*2–3 months*
5	1.66 ± 0.22 (7)	1.36 ± 0.25 (6)	1.06 ± 0.16 (5)	0.83 ± 0.22 (5)	
10	2.29 ± 0.33 (7)	2.10 ± 0.24 (6)	1.72 ± 0.16 (7)	1.42 ± 0.23 (6)	
20	3.00 ± 0.26 (7)	2.84 ± 0.58 (7)	2.61 ± 0.39 (5)	2.40 ± 0.26 (7)	
1	1.48 ± 0.26 (7)	1.15 ± 0.15 (6)	0.81 ± 0.06 (6)	0.62 ± 0.12 (4)	*18 months*
5	1.99 ± 0.20 (7)	1.53 ± 0.15 (8)	1.34 ± 0.15 (8)	1.13 ± 0.07 (8)	
10	2.13 ± 0.17 (8)	2.01 ± 0.13 (7)	1.75 ± 0.11 (9)	1.59 ± 0.16 (6)	
20	2.88 ± 0.16 (8)	2.63 ± 0.20 (7)	2.56 ± 0.17 (9)	2.21 ± 0.10 (8)	

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TABLE 2. D-GLUCOSE ILEAL ABSORPTION IN RATS OF DIFFERENT AGES DEPENDING ON SODIUM CONCENTRATION AT ONE TO TWENTY MINUTE PERIODS. EACH DATUM IS THE MEAN ± S.E.M. THE NUMBER OF ANIMALS IS IN PARENTHESIS.

		D-Glucose μmols/cm²			
Time (min.)	*Na^+ meq/l:* *154*	*77*	*38.5*	*0*	*Age*
1	2.60 ± 0.51 (5)	1.55 ± 0.39 (4)	1.15 ± 0.11 (3)	0.42 ± 0.31 (4)	
5	3.44 ± 0.80 (4)	2.39 ± 0.17 (5)	2.03 ± 0.39 (6)	0.89 ± 0.16 (5)	
10	4.01 ± 0.13 (4)	3.00 ± 0.31 (6)	2.70 ± 0.59 (4)	1.62 ± 0.40 (6)	*21–23 days*
20	4.58 ± 0.35 (6)	4.25 ± 0.35 (6)	3.62 ± 0.55 (6)	2.82 ± 0.44 (5)	
1	1.25 ± 0.23 (6)	0.93 ± 0.34 (4)	0.54 ± 0.17 (5)	0.46 ± 0.05 (5)	
5	1.36 ± 0.17 (4)	1.30 ± 0.24 (6)	0.91 ± 0.15 (6)	0.73 ± 0.23 (6)	
10	1.66 ± 0.16 (6)	1.50 ± 0.16 (6)	1.29 ± 0.12 (7)	0.97 ± 0.27 (6)	*2-3 months*
20	2.39 ± 0.27 (3)	2.26 ± 0.21 (6)	1.98 ± 0.09 (6)	1.72 ± 0.32 (7)	
1	1.42 ± 0.11 (4)	0.93 ± 0.12 (6)	0.87 ± 0.12 (5)	0.75 ± 0.19 (6)	
5	1.55 ± 0.17 (5)	1.21 ± 0.09 (6)	1.08 ± 0.14 (6)	0.92 ± 0.17 (9)	
10	1.85 ± 0.15 (6)	1.40 ± 0.15 (7)	1.36 ± 0.11 (6)	1.15 ± 0.12 (6)	*18 months*
20	2.09 ± 0.16 (8)	1.97 ± 0.18 (8)	1.90 ± 0.23 (6)	1.82 ± 0.19 (8)	

The absorption of D-Glucose was studied under sodium concentrations in the lumen of 154, 77, 38.5 and 0 meq/l and it was observed that inhibition increased when sodium concentrations decreased, though the jejunum and the ileum did not show the same degree of inhibition.

The greatest inhibition in the three groups of animals was during the first minute of the perfusion, showing a linear relationship between D-Glucose uptake and sodium concentrations in the lumen (Figures 1 and 2). In the jejunum there was not a significative difference between the slopes at the three ages studied. By contrast, in the ileum there was a statistical difference ($p < 0.05$) between the youngest rats and the two other groups. The 21–23 day old animals showed the greatest slope in the ileum.

Table 3 shows the outflux of Na from the jejunum and the ileum to the lumen after 20 minutes perfusion with the sodium free medium. There was statistical differences between the jejunum and the ileum ($p < 0.01$ at 21–23 days, $p < 0.05$ at 2–3 months and 18 months old). The greatest outflux of Na was in the jejunum of the youngest animals.

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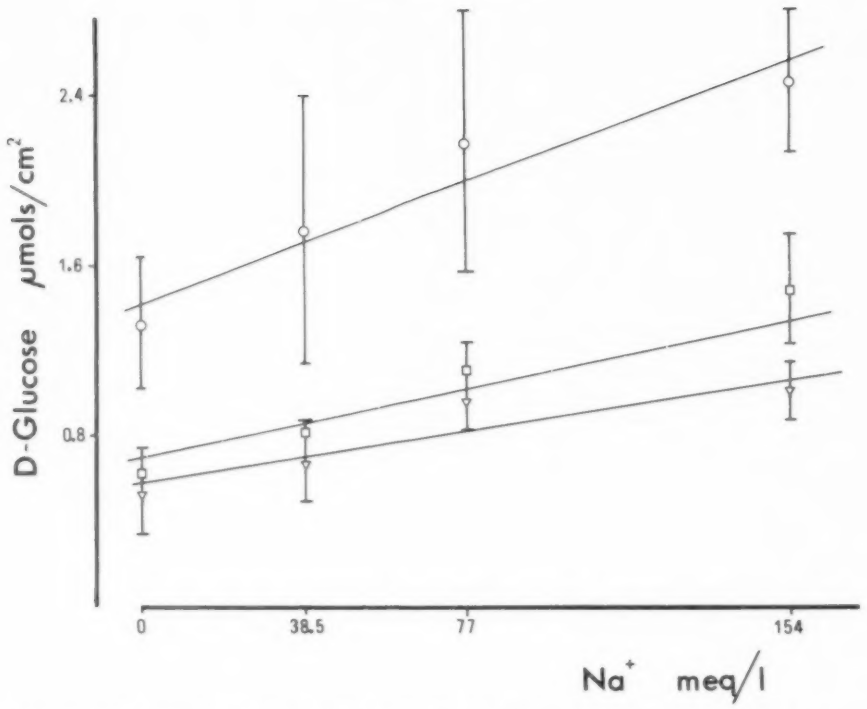

FIG. 1. Relationship between D-Glucose jejunal absorption and sodium concentration in the first minute period at different ages of the rat. (○ — ○ 21–23 days old y = 0.0073 x + 1.4380, r = 0.9619) (▽ — ▽ 2–3 months old y = 0.0037 x + 0.5520, r = 0.9557) (□ — □ 18 months old y = 0.0057 + 0.6300, r = 0.9895) Mean values ± S.E.M.

DISCUSSION

In accordance with other authors we have found a greater D-Glucose absorption in the jejunum than in the ileum (Debnam, E.S. 1979) and in the 21–23 days old animals than in the older ones (Younoszai, M.K. and Lynch, A. 1975).

When sodium was partially substituted in the medium, there was an inhibition of in-

TABLE 3. OUTFLUX OF Na^+ INTO THE LUMEN AFTER 20 MINUTES PERFUSION OF D-GLUCOSE 10 mM IN A SODIUM FREE MEDIUM AT DIFFERENT AGES. EACH DATA IS THE MEAN OF AT LEAST 6 ANIMALS. ± S.E.M.

	Na^+ (µmols/20 min./cm²)	
Age	*Jejunum*	*Ileum*
21–23 days	25.49 ± 6.06	3.04 ± 1.01
2–3 months	2.02 ± 0.05	0.50 ± 0.30
18 months	16.38 ± 1.26	5.94 ± 1.34

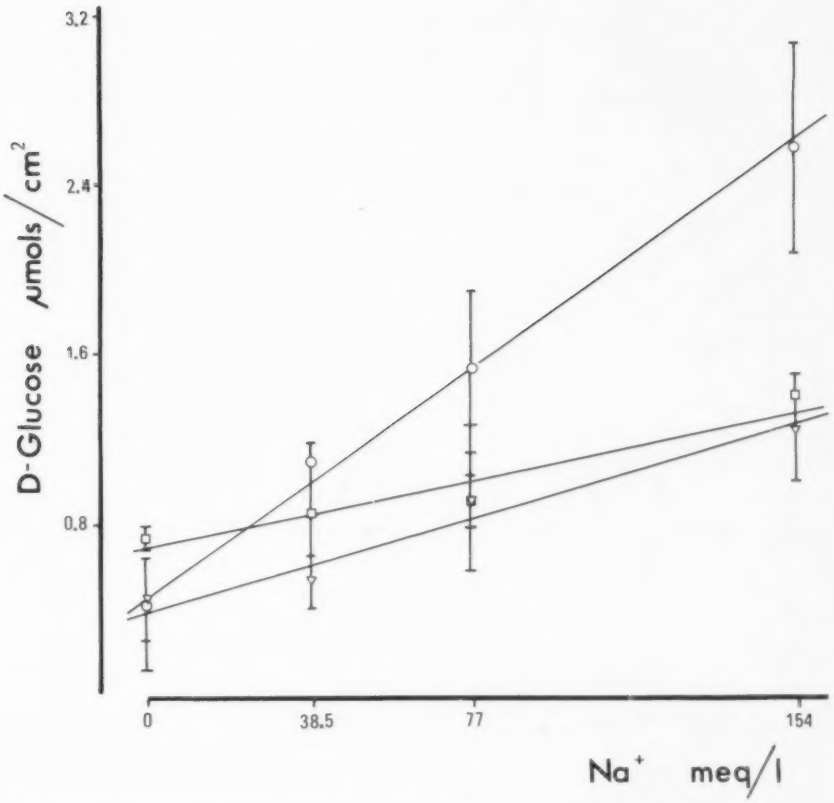

FIG. 2. Relationship between D-Glucose ileal absorption and sodium concentration in the first minute period at different ages on the rat. (○ — ○ 21-23 days old y = 0.0138 x + 0.5020, r = 0.9957) (▽ — ▽ 2-3 months old y = 0.0054 x + 0.4280, r = 0.9778) (□ — □ 18 months old y = 0.0043 x + 0.700, r = 0.9687) Mean values ± S.E.M.

testinal D-Glucose absorption that increased according with decreasing sodium concentration. Like Ortiz *et al.* (1979), we found that with one minute periods, inhibition of D-Glucose intestinal absorption was greater than in the longest periods. This author's results were for the jejunum of 2 month old rats, and our results show this effect in the three groups of animals employed in both the jejunal and ileal segments.

The greater inhibition of D-Glucose uptake in one minute periods than in longer periods could be attributed to an outflux of endogenous sodium toward the lumen observed after 20 minutes perfusion (Table 3). The outflux of sodium at different ages has been studied in experimental diarrhea only, perfusing hyperosmotic solutions and showed a greatest loss of sodium in young rats than in adults ones. (Younoszai, M.K. and Robillard, J.E. 1980).

The differences observed between the two intestinal segments and at the different stages of life (Table 3) could be attributed to differences in the characteristics of the junctions, supposing that the ouflux of sodium takes place throughout these junctions (Schultz, S.G. and Frizell, R.A. 1972). An effect of Tris-HCl on sugar absorption was excluded (Ortiz,

M. *et al.*, 1979). Lack of sodium in the perfusion medium did not entirely block absorption because D-Glucose chiefly enters the intestine by diffusion, especially with *in vivo* techniques (Robinson, J.W.L. and Antonioli, J.A. 1980).

The greater dependence on sodium concentration of D-Glucose absorption observed in the ileum of 21–23 day old rats should be attributed to the fact that in the ileum at this age active transport mechanisms play an important role (Vinardell, M.P. and Bolufer, J., in press). In the ileum the physiological concentration of sugars is smaller than in the jejunum (Ilundain, A. *et al.*, 1979). There presumably is a mechanism to absorb all the sugar present in the lumen and to prevent the possible loss of it. This mechanism is active transport.

By contrast in the jejunum the youngest rats absorbed D-Glucose in more amount than the older animals, but active transport did not play the same role as it did in the ileum as could be seen in the relationship between D-Glucose absorption and sodium concentration (Figure 1). Shepherd, R.W. *et al.*, (1980) found a decrease in glucose-coupled sodium transport in the suckling rabbit compared to corresponding transport rates in adult rabbits. This difference with our results could be attributed to the animal species.

Our present results confirmed the decreased component of active transport mechanism in older animals, especially in the ileum (Vinardell, M.P. and Bolufer, J., in press).

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HORMONAL, METABOLIC AND MORPHOLOGIC STUDIES OF AGED C57BL/6J OBESE MICE

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Abstract—Genetically obese mice (C57BL/6J-*ob*/*ob*), fed *ad libitum*, demonstrated a precipitous increase in the spontaneous death rate after 50 weeks. The first signs of morbidity were a ruffled hair coat and a progressive motor ataxia. Necropsy revealed that obese mice had pale and fatty livers, urolithiasis and grossly distended bladders. Microscopically, the hepatocellular changes observed in all aged obese mice included: a loss of orientation of hepatocytes, an enormous variability in the size of both hepatocytes and their nuclei, and an extensive deposition of both large and small lipid droplets, confirmed by an increase content of triacylglycerols. A subacute-to-chronic, multifocal, necrotizing hepatitis was also present. Kidneys from aged obese mice contained hypertrophied glomeruli and increased PAS-stained material. Tubular dilation with compaction of the tubular cells was also seen. There were no significant alterations in the microanatomy or mineralization of femurs from obese mice, yet there was a significant increase in plasma alkaline phosphatase activity. In obese mice at 62–63 weeks of age, hyperglycemia was present even in spite of hyperinsulinemia. Pituitary immunoreactive ACTH and its molar ratio to pituitary immunoreactive β-endorphin were also increased in obese mice at this age. Even though the etiology of the decreased lifespan of genetically obese mice remains uncertain, the possibility is discussed that an overall defect in the central nervous system may be involved.

INTRODUCTION

THE GENETICALLY obese mouse (C57BL/6J-*ob*/*ob*) develops a syndrome of obesity and insulin resistance which is caused by a single recessive gene (Ingalls *et al.*, 1950) and resembles noninsulin-dependent diabetes in man (Bray and York, 1979). These mice display numerous and profound hormonal and metabolic alterations (Herberg and Coleman, 1977; Bray and York, 1979). The mean lifespan for obese mice fed *ad libitum* is approximately 60 weeks which can be lengthened by caloric restriction to more than 100 weeks (Lane and Dickie, 1958). Few studies have examined the obese mouse throughout

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its lifespan (Westman, 1968). Recently, we reported that the obese mouse fed *ad libitum* is characterized by hyperinsulinemia and hyperadrenocorticism throughout its life (Garthwaite *et al.*, 1980). This longitudinal aging profile was terminated when the obese mice were 62–63 weeks of age as neurological symptoms developed, accompanied by a marked increase in mortality.

In the present study, we have examined structural, functional and biochemical changes in selected organs of the genetically obese mouse near the end of its lifespan. The investigation of the pathophysiological lesions in this well-defined mouse strain should increase our understanding of the complications associated with obesity and noninsulin-dependent diabetes in humans, in which a myriad of genetic and environmental factors interplay in the disease process.

MATERIALS AND METHODS

Animals

Male obese (C57BL/6J-*ob*/*ob*) mice and their lean controls (C57-BL/6J−?/+) were purchased as weanlings from Jackson Laboratory (Bar Harbor, ME). A group of increasingly aged mice (50–63 weeks) was developed by obtaining matched groups of at least 6 obese and 12 lean mice with known birth dates ± 3 days at approximately 1-month intervals throughout the extent of the profile (Garthwaite *et al.*, 1980). All mice were raised in a constant ambient temperature (21 ± 1°C) environment, with 12 hours light, 12 hours dark cycle (lights on from 0600–1800 hours) and *ad libitum* access to Lab Chow (Ralston Purina Company, St. Louis, MO) and tap water. They were kept in shoebox mouse cages with two obese or five lean mice per cage. All mice were weighed at weekly intervals and were examined daily for physical abnormalities. Necropsy was performed on all mice found dead during the duration of the study. The experiment was terminated within a 1-week period when the oldest group of obese mice and lean controls were 62–63 weeks of age.

At the end of the experiment, obese mice and their age-matched controls in the fed state were anesthetized (110 mg pentobarbital sodium/kg, i.p.) and exsanguinated via cardiac puncture between 0800–1100 hours. Heart, liver and then brain were sampled by freeze-clamp technique at the temperature of liquid nitrogen (Wollenberger *et al.*, 1960). Pituitaries were collected in 1 ml ice-cold O.1M HCl and the cells were disrupted for 5 seconds using a sonicator fitted with a microtip (Branson Instrument Co., Stamford, CT). Lightly heparinized plasma from blood obtained by cardiac puncture and both hind limbs were stored at −15°C. Pituitaries and brains were stored at −20°C for a period of less than 3 months until assayed.

Analytical Methods

Plasma glucose was determined enzymatically without protein precipitation by the hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) method (Bergmeyer *et al.*, 1974). Insulin was determined by radioimmunoassay using a modification of the method described by Yalow and Berson (1960). The assay standard and iodination material were highly purified porcine insulin (25.4 U/mg; lot 615-D-63-10, Eli Lily, Indianapolis, IN) generously supplied by Dr. Mary Root. The antibody was guinea pig antiporcine insulin (lot 2C-5278, Biotek Research, Inc., Shawnee Mission, KS). The assay was performed under disequilibrium conditions with [^{125}I] insulin added on day two and separation by charcoal-dextran on day three.

Corticosterone was extracted with methylene chloride and determined by radioimmunoassay using corticosterone-21-thyroglobulin antibody (Miles Laboratories, Inc., Elkhart, IN). The use of [^{3}H) corticosterone as tracer and dextran-coated charcoal to separate free from bound tracer was based on methodology described by Murphy (1967). Correction was made for extraction efficiency; the efficiency was determined in each run and averaged 85%.

The ACTH radioimmunoassay and antibody used to obtain the present data have been described previously (Hagen *et al.*, 1977). Serial dilution of a homogenate of mouse pituitary in assay buffer cross-reacted in parallel with α-ACTH-(1-24) suggesting this assay qualitatively measures mouse ACTH. β-Endorphin was estimated by radioimmunoassay based on the method of Guillemin *et al.* (1977). The antibody was obtained from rabbits which had been injected with immunoglobulin G-conjugated human β-endorphin (β_h-endorphin), and β_h-endorphin was used for standards (β_h-endorphin and antibody were generously supplied by Dr. C.H. Li, San Francisco, CA). This antibody has approximately 10% cross-reactivity with B_h-lipotropin but no cross-reactivity with ACTH, MSH, or the enkephalins. β_hEndorphin was labeled with [^{125}I] by a modification of the chloramine-T method of Hunter and Greenwood (1962). β_h-[^{125}I] Endorphin, antibody to $\beta_h$endorphin and standards or unknowns were incubated for 24 hours and then separated with 2% (w/v) charcoal coated with 1% (w/v) bovine serum albumin in assay buffer. All samples were assayed in triplicate in a single assay. The protein content of the pituitary homogenate was determined by the method of Waddell (1956) using crystalline bovine serum albumin as the standard. Brain serotonin was determined by the technique of Curzon and Green (1970).

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Triacylglycerol content of homogenates of frozen heart or liver powder in water was determined by the method of Laurell (1966). Plasma samples were also extracted and saponified as described by Laurell (1966) although the triacylglycerol-glycerol was quantitated by a fluorometric adaptation of an enzymatic assay for glycerol (Wieland, 1974).

Activity of alkaline phosphatase in plasma was assayed by determining the amount of 4-nitrophenol liberated from 4-nitrophenol phosphate at 25°C with a recording spectrophotometer at 405 nm and is reported in international units per liter of plasma (Walter and Schütt, 1974).

Hind limbs from obese and lean mice were stored at −15°C until the femur was dissected free from adhering tissue while the limb was still frozen. After drying at 110°C for 18 hours, each bone sample was wrapped individually in a Whatman No. 1 filter paper disc (9 cm diameter) and extracted with diethyl ether for 48 hours. The samples were then air dried for at least 24 hours, transferred to tared porcelain crucibles and dry ashed at 550°C for 20 hours in a muffle furnace. The crucibles were cooled in a dessicator and reweighed. The sample was then prepared by dissolving the ash in concentrated HCl, diluted in the presence of lanthanum and the calcium was assayed in a Model 303 Atomic-Absorption Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) in an air-acetylene flame. Bone phosphate was assayed by reduction of the phosphomolybdate complex with ascorbic acid (Ames, 1966). The ash sample used in analysis of calcium was employed for phosphate analysis without interference from the lanthanum present. Plasma calcium was also determined by atomic absorption spectrophotometry with care to avoid precipitation of the proteins in the plasma during sample preparation. Inorganic phosphate content of plasma (Ames, 1966) was assayed in neutralized, perchloric acid filtrates.

Histology and Ultrastructure

Organs obtained from both obese mice and age-matched lean controls at necropsy were fixed in 10% (v/v) phosphate-buffered formalin for 24 hours, processed for routine paraffin embedding and sectioned at 5 μm. The sections were stained with hematoxylin and eosin, and examined. Femurs were obtained intact and surrounding soft tissues were removed prior to fixation in 10% phosphate-buffered formalin. Bone specimens were fixed for 48 hours, decalcified in 0.5M EDTA, embedded in paraffin, and 5 μm sections were microscopically evaluated.

Statistics

Differences between obese mice and age-matched lean controls at 62–63 weeks of age were analyzed by Student's *t* test for unpaired data (Steel and Torrie, 1960).

RESULTS

Profile of Aged Obese Mice

At approximately 50 weeks of age, obese mice demonstrated abnormalities in physical appearance which progressed over the next 4- to 8-week period. A ruffled hair coat was constantly present. An advancing, worsening motor ataxia also characterized aged obese mice. A precipitous increase in mortality occurred in obese mice of more than 50 weeks of age with only a few animals surviving at the termination of the longitudinal profile. The decreased survival of a group of genetically obese mice that would have been 58–59 weeks of age when the longitudinal aging profile was terminated is illustrated in Figure 1. All obese and lean mice older than 50 weeks were examined daily and a complete necropsy was performed on all mice found dead.

Macroscopic Findings

At necropsy, all obese mice older than 50 weeks had enlarged, pale and uniformly fatty livers. Almost all of the aged obese mice had urolithiasis as evidenced by copious deposits of small white concretions of unknown composition in the bladder. Bladders of aged obese mice were also grossly distended. Brain, heart, lung, kidneys as well as the lymphoreticular system (spleen, peripheral lymph nodes, Peyer's patches and an involuted thymus) were unremarkable. No abnormalities were noted in age-matched lean control mice killed for comparison with obese mice.

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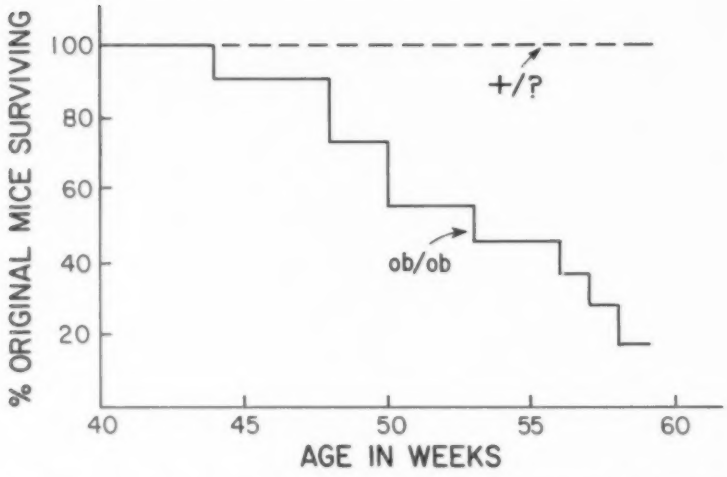

FIG. 1. Survival of genetically obese mice and lean controls. Groups of 11 obese (C57BL/6J-*ob/ob*) and 7 lean (C57BL6J−?/+) mice were followed daily until the remaining mice were 58–59 weeks of age when they were sacrificed at the termination of the longitudinal profile.

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Histopathology

Severe fatty deposition and degenerative changes were present in the livers of all obese mice older than 50 weeks (Figure 2A–C). The hepatocellular changes included a loss of orientation of hepatocytes. They no longer formed sheets of cells and lacked orientation around the central veins and portal triads. Furthermore, an enormous variability in the size of both hepatocytes and their nuclei was also seen. There was also an extensive content of both large and small lipid droplets in the cytoplasm (Figure 2C).

In addition to the augmented lipid deposition (Figure 2C), a subacute-to-chronic, multifocal, necrotizing hepatitis was observed in all obese mice older than 50 weeks in age (Figure 2D). Such lesions were never seen in livers of age-matched lean controls. The inflammatory process in the livers of obese mice was considered moderate and not cirrhotic since neither nodular regeneration nor bands of fibrous tissue were found.

Kidneys from aged obese mice contained glomeruli that were hypertrophied and had increased PAS-stained material when compared to age-matched lean controls (Figure 3A,B), Tubular dilation with compaction of the tubular cells was also seen in obese mice. These animals were also found to have urolithiasis and grossly distended bladders.

Metabolic Parameters

The determinations made in obese mice at 62–63 weeks of age were limited by the increasing mortality that occurred (Figure 1). The surviving obese mice at this age had an average body weight that was twice that of the lean control (Table 1; $p < .001$). Absolute liver weight was also significantly increased in obese mice (Table 1). The relative liver mass per 100g body weight was, however, similar in obese mice and their lean controls. Heart weight in the obese mouse at 62–63 weeks of age was significantly greater than that of the lean control (Table 1; $p < .05$). But when expressed per 100g body weight, the

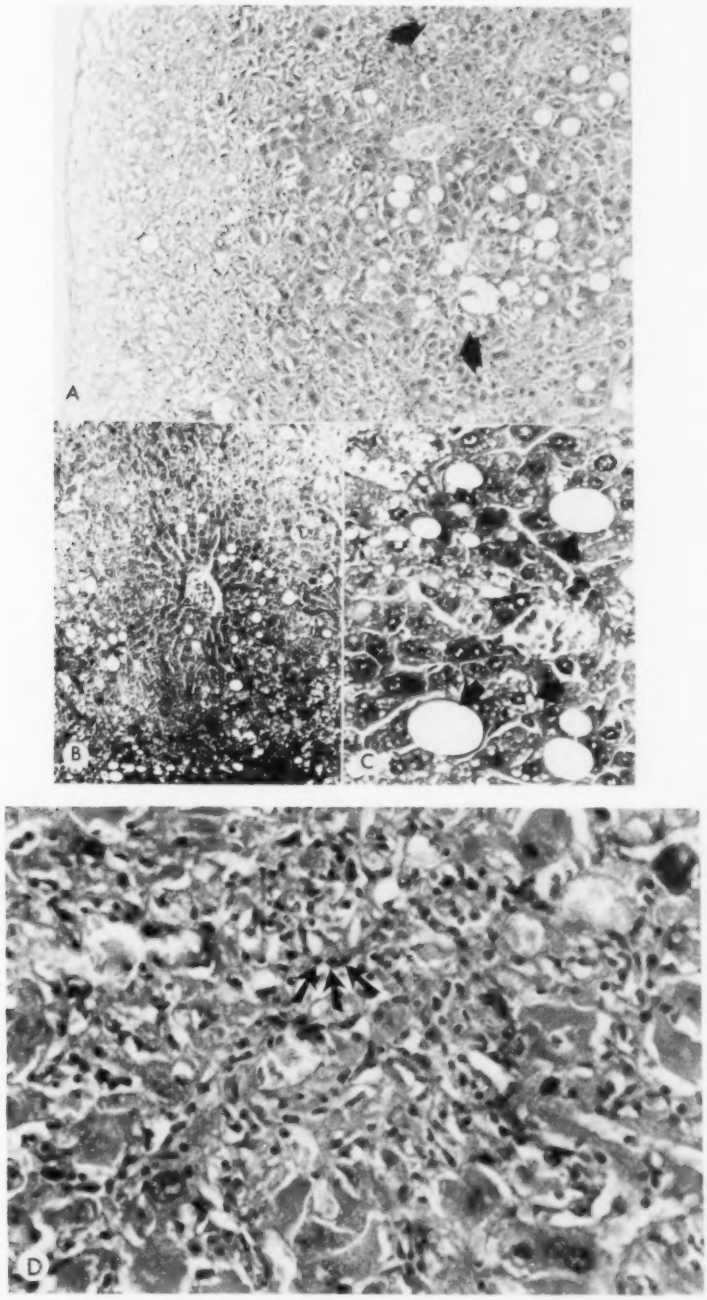

FIG. 2. Changes in the liver of a 62–63 week-old obese mouse. A—Loss of orientation of hepatocytes in the form of sheets (arrows) (H and E, × 110). B—Enormous variability in the size of both hepatocytes and their nuclei (H and E, × 550). C—Extent of lipid deposition with both large and small droplets in the cytoplasm (arrows) (H and E, × 710). D—Multifocal, necrotizing hepatitis and increased fibroblastic activity (arrows) (H and E, × 1000).

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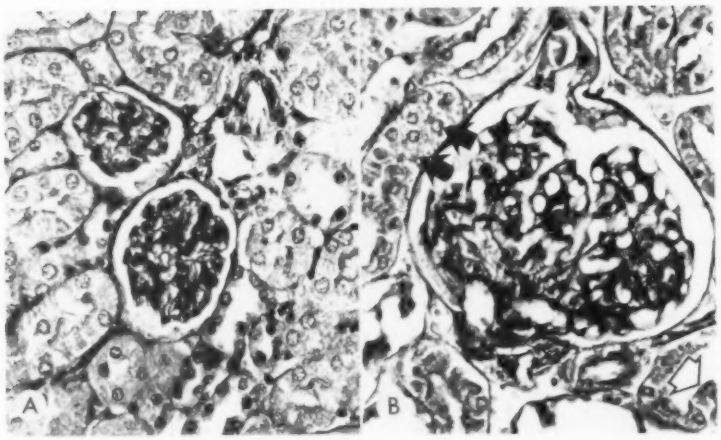

FIG. 3. PAS-stained section of kidney from a 62–63 week-old lean mouse (A) demonstrating normal glomeruli and tubules. The kidney from an age-matched obese mouse (B) shows enlarged glomeruli, tubular dilation (open arrows) and compressed tubular cells (solid arrows). Increased amounts of PAS-positive material were also observed although it is not evident in the black and white photomicrographs (× 710).

relative heart weight of obese mice was dramatically lower than the lean control. The plasma glucose concentration in obese mice was two-fold higher than that seen in lean mice ($p < .001$) even though the plasma insulin was elevated in the aged obese mice, indicative of insulin resistance.

In agreement with severe hepatocellular fatty deposition found in aged obese mice (Figure 2A–C), hepatic triacylglycerol content in obese mice at 62–63 weeks of age was increased four-fold when compared to age-matched lean controls (Table 2). Even though the hearts of aged obese mice appeared histologically normal, myocardial triaclyglycerol content was almost two-fold greater than that of lean controls (Table 2). In spite of ac-

TABLE 1. BODY, LIVER AND HEART WEIGHTS, PLASMA GLUCOSE AND INSULIN IN OBESE AND LEAN MICE AT 62–63 WEEKS OF AGE.*

	Obese	*Lean*
Body weight (g)	79.2 ± 2.3(4)	37.6 ± 0.5(15)
Liver weight		
Absolute (g wet)	3.87 ± 0.43(4)	1.70 ± 0.05(15)
Relative (g liver/100g b.w.)	4.89 ± 0.48(4)	4.50 ± 0.08(15)
Heart weight		
Absolute (mg wet)	185 ± 12(4)	151 ± 5(15)
Relative (mg heart/100g b.w.)	234 ± 15(4)	403 ± 11(15)
Plasma		
Glucose (mmol/l)	20 ± 3.5(4)	10.2 ± 0.2(20)
Insulin (μU/ml)	127, 137**	27 ± 3(20)**

*Values are the mean ± S.E.M. except for plasma insulin in the obese group where individual determinations on two mice are given. The number of animals is indicated in parentheses.

**Obese and lean mice were 57–63 weeks of age.

TABLE 2. CONTENT OF TRIACYLGLYCEROLS IN PLASMA, LIVER AND HEART VENTRICULAR TISSUE IN OBESE AND LEAN MICE AT 62–63 WEEKS OF AGE.*

	Obese	*Lean*
Plasma (μmol/ml)	0.12 ± 0.06	0.47 ± 0.04
Liver (μmol/g wet wt)	74, 106	23.2 ± 3.3
Heart (μmol/g wet wt)	5.9 ± 0.7	3.1 ± 0.5

*Values are the mean ± S.E.M. for tissues from three obese mice except where indicated, and for four lean controls.

cumulation of neutral lipid in both the heart and liver of aged obese mice, plasma content of triacylglycerol was reduced to a value that was only one-third of the age-matched lean control (Table 2).

Bone Structure, Mineralization and Histology

At necropsy, femurs of aged obese mice could be bent without breaking. This response was distinctly different from femurs of lean mice which fractured when pressure was applied. However, bone mineral content of femurs from both obese and lean mice at 62–63 weeks of age was similar (Table 3). Also, plasma Ca and P_i values were not different in obese and lean mice at this age. Yet, plasma alkaline phosphatase activity was elevated five- to six-fold in obese mice when compared to that of age-matched lean controls (Table 3).

Microscopic examination of bone from obese and lean mice demonstrated osteocytes and vascular channels which were randomly oriented in a matrix consisting of non-Haversian lamellae (Figure 4A,B). Osteoclasts were rarely observed and matrical staining was identical in bone sections of obese and lean mice. Extensive lipid deposits were scattered throughout the bone marrow of obese mice when compared to that of the age-matched lean controls. (Figure 4A,B).

TABLE 3. BONE MINERAL AND PLASMA CALCIUM, INORGANIC PHOSPHATE AND ALKALINE PHOSPHATASE IN OBESE AND LEAN MICE AT 62–63 WEEKS OF AGE.*

	Obese	*Lean*
Femur		
Ash (% dried, fat-free femur)	58.5 ± 3.5	58.3 ± 0.3
Ca (% of femur ash)	35.8 ± 1.7	31.1 ± 0.9
P (% of femur ash)	19.2 ± 0.9	20.0 ± 0.9
Plasma		
Ca (mmol/l)	1.93 ± 0.31	1.77 ± 0.29
P_i (mmol/l)	2.26 ± 0.66	2.24 ± 0.18
Alkaline phosphatase (IU/l)	82.0 ± 20.5	14.9 ± 3.5

*Values are the mean ± S.E.M. of analyses for three obese mice and four mice in the lean control group.

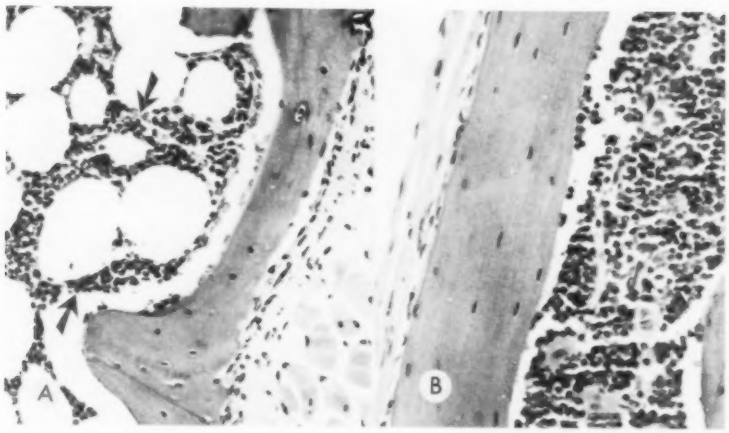

FIG. 4. Decalcified bone sections from the femur of a 62–63 week-old obese mouse (A) and that of an age-matched lean control (B demonstrating the non-Haversian lamellae comprising the matrix. Extensive lipid deposition scattered throughout the bone marrow of obese mouse (A, arrows) (H and E, × 285).

Pituitary and Brain

Since a progressive motor ataxia occurred with high incidence in obese mice older than 50 weeks, several determinations were made on both the pituitary and brain (Table 4). Total pituitary protein in 62–63 week-old obese mice was increased when compared to age-matched lean controls though the difference was not statistically significant. The ACTH content was increased yet immunoreactive β-endorphin was significantly diminished in the pituitary of the aged obese mouse. As obese mice approached the end of their lifespan, the molar ratio of pituitary ACTH to β-endorphin was elevated significantly (Table 4, $p < .05$) due to a 15-fold increase in immunoreactive ACTH compared to only five-fold increase in immunoreactive β-endorphin. Plasma corticosterone levels were also higher in aged obese mice but this difference was not statistically significant when compared to those of the lean controls.

TABLE 4. PITUITARY PROTEIN, ACTH AND β-ENDORPHIN, PLASMA CORTICOSTERONE AND BRAIN WEIGHT AND SEROTONIN IN OBESE AND LEAN MICE AT 62–63 WEEKS OF AGE.*

	Obese	*Lean*
Pituitary		
Protein (μg)	540 ± 70 (4)	450 ± 30 (18)
ACTH (ng/mg protein)	730 ± 400 (4)	45 ± 6 (18)
β-Endorphin (pmol/μg protein × 10^4)	870 ± 300 (4)	160 ± 20 (20)
Molar ratio ACTH to β-endorphin	0.25 ± 0.13(4)	0.07 ± 0.02(16)
Plasma corticosterone (μg/dl)	14.1 ± 4.8 (3)**	5.9 ± 0.5 (12)**
Brain		
Weight (mg wet wt)	360 ± 20 (4)	380 ± 10 (20)
Serotonin (μg/g wet wt)	11.4 ± 0.3 (3)	8.5 ± 0.5 (5)

*Values are the mean ± S.E.M.; the number of animals is indicated in parentheses.
**Obese and lean mice at 57–63 weeks of age.

Although brain weights were similar in aged obese mice and their lean controls, serotonin concentration in the brain of the obese mouse at 62–63 weeks of age was increased (Table 4, $p < .01$).

DISCUSSION

The obese hyperglycemic mouse (*ob/ob*) has been used as a model for human obesity and adult-onset (noninsulin-dependent) diabetes mellitus (Herberg and Coleman, 1977; Bray and York, 1979). Although studies of the endocrine and metabolic status of the obese mouse are plentiful, (Herberg and Coleman, 1977; Bray and York, 1979), including our recent examination of 4- to 5-month-old mice (Garthwaite *et al.*, 1979), most have restricted their observations to the dynamic phase of obesity up to a body weight of 40–50 g. In a few studies (Lane and Dickie, 1958; Christophe *et al.*, 1959; Westman, 1968; Herberg *et al.*, 1970), obese mice have been examined that were greater than 12 months in age, including our recent investigation of their endocrine status from weaning to an age approaching its lifespan (Garthwaite *et al.*, 1980). A precipitous increase in mortality occurred in obese mice more than 50 weeks of age with only a few mice surviving until the termination of the longitudinal profile when the oldest mice were 62–63 weeks of age. The mean lifespan for obese mice (C57BL/6J-*ob/ob*) fed *ad libitum* has been previously reported to be 457 days with a range of 167–667 days, (Lane and Dickie, 1958), which is similar to the findings in the present study with the same strain of mice. The lifespan of (C57BL/6J-+/+ mice has been found to be about 820 days (Goodrick, 1975). Thus, mice with the homozygous mutant gene *ob* on the C57BL/6J background have a lifespan that is shortened by approximately 50% when compared to C57BL/6J-+/+ mice. Westman (1968) found the *ob* mice from a noninbred colony maintained at the Department of Histology, University of Uppsala, Sweden, had a lifespan only a few months shorter than lean littermates, most of them dying between 20 and 24 months of age. The difference in the lifespan of obese mice originating from Jackson Laboratory and those from the Uppsala Colony might be due to: the different genetic backgrounds, that is the BL/6J at the Jackson Laboratory and the V stock in Uppsala; dissimilar breeding schemes, that is inbreeding (Jackson Laboratory) and non-inbreeding (Uppsala); or the diversity in environmental factors between the two locations.

At necropsy, aged obese mice in the present study had urolithiasis and grossly distended bladders. Kidneys from the same animals contained glomeruli that were hypertrophied and had an increase in PAS-stained material. Tubular dilation was also seen but no prominent lipohyalin glomerular lesions were observed in the present study. Nathorst-Windahl and Hellman (1964) reported that such lesions occur in aged (22–26 months old) obese hyperglycemic mice of the Swedish colony. In any case several renal functions, including an inhibition of transport capacity, have been found to be depressed even in obese mice with the mutant gene *ob* on the C57BL/6J background (Johnson *et al.*, 1975). The possibility exists that alterations in the microscopic structure of the glomeruli and the tubular tissue is due to an acceleration of the aging process favored by the obese mouse syndrome (Nathorst-Windahl and Hellman, 1964).

Aged obese mice had femurs that could be bent without breaking yet the bone was calcified normally (Table 3) and microscopically identical to that of lean mice (Figure 4A,B). Likewise, plasma Ca and P_i values were similar in obese and lean mice at 62–63 weeks of age. Reduction in bone density and mass has been found in human diabetics

(Levin *et al.*, 1976; Tamayo *et al.*, 1981) as well as in experimental alloxan (Ramamurthy *et al.*, 1973) and streptozotocin (Brown and Jowsey, 1977) diabetes. Likewise, a reduction of bone mass has been reported to occur in 22-week old C57BL/6J-*ob/ob* mice yet neither bone nor plasma calcium content differed from that of the lean control (Kawashima and Castro, 1981). In the present study, plasma alkaline phosphatase was elevated in aged obese mice. Susceptibility of plasma alkaline phosphatase in obese mice to heat inactivation, urea denaturation, and inhibition to l-phenylalanine, l-homoarginine and l-p-bromotetramisole was consistent with that of the skeletal isoenzyme (Menahan, unpublished observations). An increased activity of bone isoenzyme of serum alkaline phsophatase has also been found in human diabetics (Štěpán *et al.*, 1980). Defects in bone metabolism in both human and experimental diabetes resemble those found in osteoporosis (Ramamurthy *et al.* 1973; Brown and Jowsey, 1977; Štěpán *et al.*, 1980; Tamayo *et al.*, 1981). Even though the factors responsible for these changes are not exactly known, lα-hydroxyvitamin D_3 treatment alleviated the reduction of bone mass usually found in obese mice (Kawashima and Castro, 1981).

In the present study, all obese mice older than 50 weeks in age had pale livers with marked lipid deposition, and a loss of hepatocyte orientation. A subacute-to-chronic, multifocal, necrotizing hepatitis of unknown etiology was also present. Since serological studies were not performed, it can not be ruled out that such lesions were the result of a latent mouse viral hepatitis infection. Lipid accumulation in the myocardium of obese mice was also seen, in agreement with the ultrastructural findings of Giacomelli and Wiener (1979). Yet, plasma content of triacylglycerols was markedly reduced in obese mice. It has been shown that the flux of plasma triacylglycerols was enhanced in obese mice, and that the extra triacylglycerol was formed in the liver both from plasma free fatty acids and by snythesis *de novo* of fatty acids from carbohydrate (Salmon and Hems, 1973). The newly synthesized hepatic triacylglycerols were found to be not released quickly enough to prevent fat accumulation in the liver of the obese mouse (Assimacopoulos-Jeannet *et al.*, 1974). An increased rate of triacylglycerol removal by peripheral tissues in obese mice due to enhanced activity of lipoprotein lipase activity (Enser, 1972) could be responsible for the reduction of plasma triacylglycerols observed in the present study. In particular, an enhanced activity of lipoprotein lipase in cardiac muscle (Enser, 1972) could accelerate triacylglycerol removal in obese mice and thereby contribute to myocardial lipid accumulation found in these mice.

The macroscopic findings at necropsy of obese mice and the subsequent histopathological examination did not seem to account for the progressive motor ataxia and death of these animals. Several parameters, however, did change in obese mice older than 50 weeks of age. In the present study, the degree of hyperglycemia, that is insulin resistance, deteriorated in the obese mouse as its life expectancy was approached in spite of hyperinsulinemia. It is well known that marked hyperglycemia first develops in obese mice during the period of rapid gain in body weight but improves dramatically after five months of age (Christophe *et al.*, 1959; Westman, 1968; Herberg *et al.*, 1970; Garthwaite *et al.*, 1980). Thus, the insulin resistance present in the young obese mouse improves in the mature animal, yet is impaired again as life expectancy is approached.

Pituitary immunoreactive ACTH has been shown to be markedly elevated in obese mice throughout their lifespan (Garthwaite *et al.*, 1980) yuet a further increase occured in animals at 62–63 weeks of age. The molar ratio of immunoreactive ACTH to immunoreactive β-endorphin in the pituitary was also increased in aged obese mice largely

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due to an elevation in ACTH. This altered ratio could result from altered synthesis, processing, or secretion of any of the precursor or completed peptides which bind to the antibodies used. Abnormalities in the structure of the central nervous system (CNS) have also recently been described in obese mice (Bereiter and Jeanrenaud, 1979). Several pathophysiological features of the mature *ob/ob* mouse syndrome suggest that a generalized CNS disorder exists including: hypogonadism and sterility, reduced growth hormone and prolactin levels, and also a thermoregulatory defect (Bereiter and Jeanrenaud, 1979; Bray and York, 1979). It is appealing to suggest that a progressive CNS dysfunction may be involved with the premature mortality observed in obese mice in the present study. If that is the case, caloric restriction which lengthens the lifespans of obese (C57BL/6J-*ob/ob*) mice to greater than 26 months (Lane and Dickie, 1958) should interact to lessen or prevent deviations that usually occur in the central nervous system of mice fed *ad libitum*.

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BIOLOGICAL AGE AND ITS ESTIMATION. III. INTRODUCTION OF A CORRECTION TO THE MULTIPLE REGRESSION MODEL OF BIOLOGICAL AGE AND ASSESSMENT OF BIOLOGICAL AGE IN CROSS-SECTIONAL AND LONGITUDINAL STUDIES

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Abstract—In order to eliminate systematic error inherent in assessment of an individual biological age (BA), a correction is suggested for the multiple regression model of BA. The larger the difference between an individual's chronological age and middle age of the sample, the greater the correction value. BA estimates of apparently healthy people of 60 to 100 years were assessed using measurements of three physiological indices: hand grip strength, short-time memory, and vibrotactile sensitivity. Introduction of the correction allowed comparison of BA estimates of the same individual obtained from repeated observations. Preliminary results show there are at least four types of the BA changes with advancing age.

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INTRODUCTION

MULTIPLE REGRESSION analysis has been used for assessment of biological age on the basis of several indices. The method was recognized as applicable for detection of some differences in the mode of aging of human subpopulations which differ from the main population in health status (Furukawa *et al.*, 1975; Webster and Logie, 1976). This approach has also given results in estimating the efficiency of various influences on aging in experimental animals (Dubina *et al.*, 1979; Skalicky *et al.*, 1980).

In the works cited, the mean biological ages of experimental and control groups of laboratory animals were compared; in the case of man the sign of the biological age deviation ("younger" or "older") was determined for all the representatives of the subpopulation considered as compared with the main population. With such an approach a systematic error, which is a property of linear regression manifesting itself by distorted estimation of biological age at the regression edges (Webster and Logie, 1976; Dubina *et al.*, 1983), does not significantly interfere since it is equally present in the estimates of the groups or subpopulations compared. When an individual's exact biological age is required and especially when it is necessary to assess it periodically in longitudinal studies, the distortion can be a serious obstacle to the use of multiple regression analysis.

In the previous paper (Dubina *et al.*, 1983) it was demonstrated with rats that the magnitude of the error in the biological age assessed using multiple regression analysis depended upon the individual's chronological age and on consistency of the regression considered with a linear model. In the present paper a correction is suggested for the multiple regression model of biological age. Introduction of the correction is demonstrated by calculating biological age of apparently normal persons at the age of 60 to 100 years examined within the program of longitudinal studies.

METHODS

Subjects

In the present work use has been made of the data obtained from observations on 250 apparently healthy elderly men and women at the age of 60 to 100 years. These observations were carried out at the Institute of Gerontology (Kiev) for more than ten years.

The observations followed a definite program which included annual observations in a hospital or in an outpatient department (Mints, 1980). The complete examination program comprised 21 physiological, biochemical and psychological indices; however, not all of the tests were performed in each of the examinations. This fact sharply reduces the volume of a sample and the number of parameters which could be used for biological age estimation. Moreover, some of the indices were rejected in the preliminary analysis because of a low correlation with age.

In this study the following parameters have been used as biological age indices: hand grip strength measured by a dynamometer (D); a short-time memory estimated as verbal memory scores (M) and the level of vibrotactile sensitivity measured as a threshold to vibratory stimuli of the right hand (V). All the parameters were measured with standard methods (Mints, 1980).

Of the total number of women only 110 examinations were chosen for inclusion in the computer program; all three parameters enumerated have been measured in them. Among the men, all the observations with the three indices were used (310 examinations). Complete characterization of the sample in relation to the age and the number of observations is given in Table 1.

Calculation of biological age

Biological age of any individual at the time of examination was calculated from the multiple regression equation which is a statistical relation between the age and the three measured parameters in the population under study. Consistency of the theoretical concepts on biological age with the multiple regression model and the calculation method had been described earlier (Dubina *et al.*, 1983).

Although for composing the equations the only data from examination of 63 women and 100 men had been used, many of them were subsequently examined again. Such repeated examinations can be considered as independent in cross-sectional studies, accordingly the multiple regression equations have been composed using the examination data for 110 women and 310 men. Table 2 contains the correlation coefficients of the measured

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TABLE 1. AGE, SEX AND EXAMINATION NUMBER OF THE EXAMINED INDIVIDUALS

	*Age groups**			
	60–74 years	*75–89 years*	*90–100 years*	*Total*
		Women		
The number of persons examined	22	23	18	63
Of them are examined				
once	9	6	7	22
twice	9	15	11	35
thrice	4	2	0	6
Total examination number	39	42	29	110
		Men		
The number of persons examined	49	25	26	100
Of them are examined				
once	1	3	8	12
twice	6	2	13	21
thrice	13	10	4	27
four times	18	7	1	26
five times	11	2	0	13
six times	0	1	0	1
Total examination number	179	81	50	310

*According to the age at the first examination

TABLE 2. A CORRELATION MATRIX FOR 110 EXAMINATIONS OF WOMEN AND 310 EXAMINATIONS OF MEN

	Age	*Hand grip strength*	*Memory*	*Vibrotactile sensitivity*	
Age		−0.736	−0.601	0.698	
Hand grip strength	−0.759		0.606	−0.492	*Women*
Memory	−0.548	0.554		−0.494	
Vibrotactile sensitivity	0.591	−0.489	−0.500		
		Men			

indices with age and with one another, the coefficients being used for calculation of the multiple regression parameters.

For 110 women (mean age is 80.8 years) the following significant ($p < 0.01$) multiple regression equation has been obtained:

$$BA_i = 84.30 - 0.639 \cdot D - 0.086 \cdot M + 0.641 \cdot V \quad (I)$$
$$(R = 0.836;\ F = 82.1 \text{ with d.f. } 106 \text{ and } 3)$$

The equation for 310 men (mean age is 76.6 years) is highly significant ($P < 0.001$):

$$BA_i = 93.02 - 0.561\ D - 0.096 \cdot M + 0.435 \cdot V \quad (II)$$
$$(R = 0.804;\ F = 186.1 \text{ with d.f. } 306 \text{ and } 3)$$

In what follows the individual biological age calculated from equations (I) and (II) is denoted by BA_i.

RESULTS

Deviation of BA means for groups of individuals at the same age from theoretical predictions on the basis of the biological age concept

In the left-hand column of Table 3 are presented BA means for the groups of women and men of the same age; the individual BA were calculated with equations (I) and (II).

TABLE 3. MEAN BIOLOGICAL AGE OF THE GROUPS OF INDIVIDUALS AT THE SAME AGE IN RELATION TO THE ASSESSMENT METHOD

Chronological age of the group, years	*Number of persons*	*Biological age, years, M ± se*	
		calculated with the ordinary method	*calculated with the correction*
	Women		
64–65 (64.4 ± 0.18)[1]	9	69.6[3] ± 1.85	64.7 ± 1.83
80–81 (80.5 ± 0.19)	8	80.9 ± 1.20	80.8 ± 1.23
93–94 (93.4 ± 0.15)	11	90.6[3] ± 1.02	94.5 ± 1.02
	Men		
65	13	70.4[4] ± 1.34	66.3 ± 1.33
78	13	76.5 ± 1.55	76.9 ± 1.55
94	8	88.6[2] ± 2.40	94.7 ± 2.40

[1]Figures in parentheses are M ± se.

Significance of the difference by Student's *t*-test as compared with the chronological age:

[2]$p < 0.05$

[3]$p < 0.02$

[4]$p < 0.001$

For the groups of women of 80 to 81 years and of men at the age of 78 (the centers of the respective regressions) BA means almost coincide with their chronological ages. In the groups with chronological age of 64–65 years ("young" members of the sample) BA means are reliably higher than the chronological age, whereas BA means at the age of 93–94 ("old" members of the sample) are essentially lower than the chronological age. This divergence of mean BA for the group of individuals at the same age from the chronological age is a contradiction to the basic concepts of biological age.

The distortion of BA means at the regression edges implies that individual BA estimates of persons whose chronological age places them at the regression edges are erroneous, too. Figure 1 shows that individual BA estimates of the women calculated in accordance with the multiple regression model lie about the line crossing the coordinate origin (an ideal line) only in the central part of the plot. Most of the points in the first ("younger") half of the regression are above this line and those in the second ("older") part of the plot fall below it.

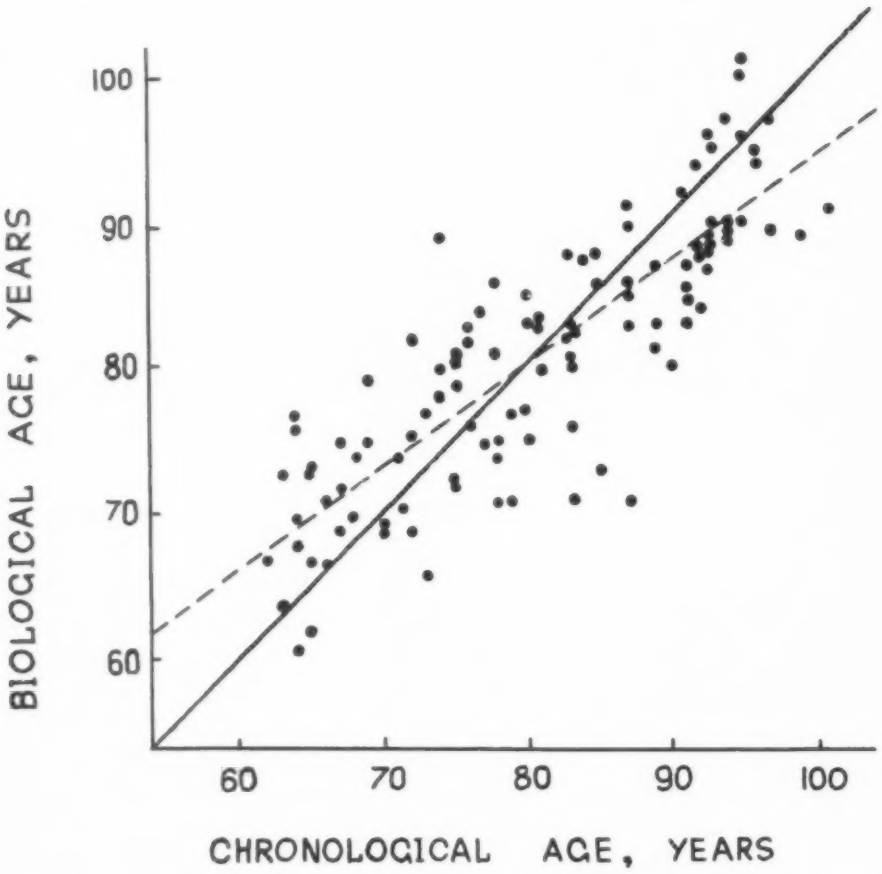

FIG. 1. Individual biological age values for women of 60 to 100 years. The values were calculated with equations (I) (*see text*). The dash-line shows linear relation between biological (BA_i) and chronological (y_i) ages, which is analytically expressed by $\mathbf{BA_i = 24.3 + 0.699\ y_i}$.

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Derivation of the correction

The concept of biological age is based on the idea that aging rate of the representatives of one species varies over certain ranges. In every population of individuals at the same age there are persons whose aging deviates from the average standard. Biological age of the individuals with a moderate aging rate coincides or is close to their chronological age. Biological age of the population members whose aging rates are slower or quicker is respectively lower or higher than their chronological age. It follows from the above that mean biological age of all the individuals at the same age should be equal to their chronological age.

When individual biological age is calculated following the multiple regression analysis, the theoretically predicted coincidence of BA mean for a group of individuals at the same age with their chronological age is observed only at the regression center, whereas at the edges we can see the effect of "narrowing": underestimated BA means for the upper regression edge and overestimated BA means for the lower edge. This effect was shown earlier for people and laboratory animals (Webster and Logie, 1976; Dubina *et al.*, 1983).

Let us consider a sample from a hypothetic population, in which biological age of the individuals is calculated on the basis of the multiple regression model. The relations between the calculated (biological) and chronological age of an individual are expressed by the equation

$$\mathbf{BA_i = a + by_i}$$

where y_i is the chronological age of an individual; BA_i is the biological age of this individual; a and b are coefficients.

These relations are shown in Figure 2 where the line AA_i is a plot of the above equation and the coefficient b is equal to the tangent of the line AA_i slope to the horizontal axis.

The individual BA values of the persons at the age of y′ range about some mean value $\overline{BA}'$ which lies on the line AA_i and is equal to the section OB. Meanwhile, according to the concept of biological age a mean biological age of individuals at the chronological age of y′ should be equal to the section OC = y′. The difference between the theoretical and multiple regression averages may be written as

$$\mathbf{z' = OC - OB}$$

Since OC = y′ and OB can be expressed as the sum of OD and BD, and OD = $\bar{y}$, BD = B_iD_i = $MD_i \cdot b$ (from the triangle MB_iD_i) we get

$$\mathbf{z' = y' - [\bar{y} + (y' - \bar{y}) \cdot b] = (y' - \bar{y}) \cdot (1 - b)}$$

where $\bar{y}$ is the chronological age average for the whole sample considered (the regression center). Then the mean biological age of a group of individuals at the age of y′ according to the concept ($\bar{Y}'$), may be written as the sum of $\overline{BA}'$ and the correction z′

$$\mathbf{\bar{Y}' = \overline{BA}' + z'.}$$

Since $\mathbf{\overline{BA}' = \frac{\Sigma BA_i'}{n}}$,

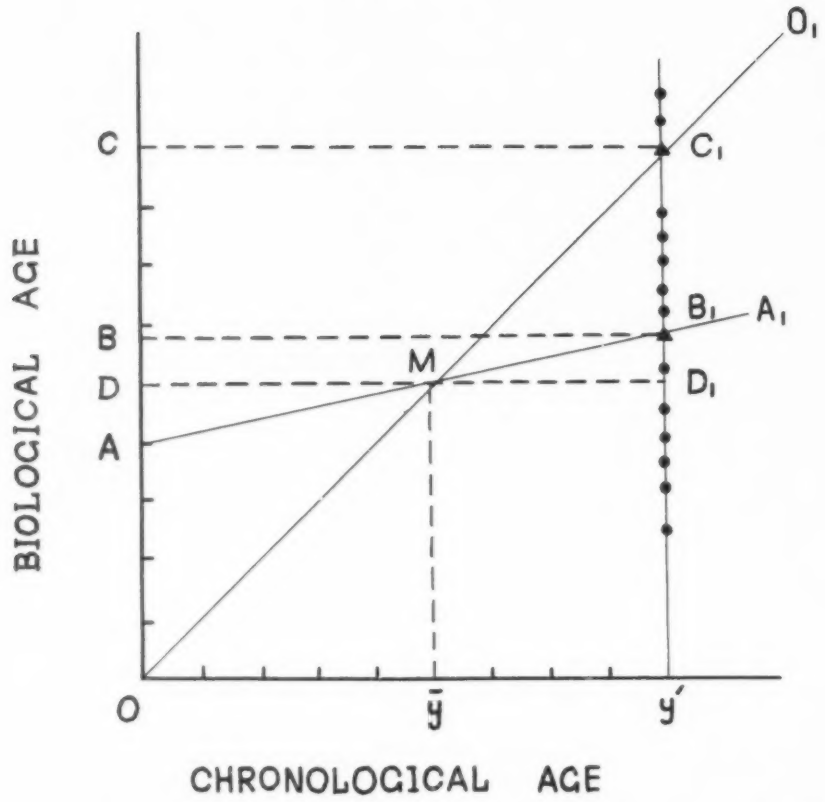

FIG. 2. Interrelation between biological and chronological ages. The derivation of the correction see in the text.

$$\text{then} \quad \overline{BA'} + z' = \frac{(BA'_1 + z') + (BA'_2 + z') + \ldots (BA'_n + z')}{n}.$$

It follows from the above that if the correction z′ is introduced to assess the "true" (consistent with the theory) biological age mean for a group of individuals at the age of y′, the "true" biological age of every individual in that group can also be assessed with the correction z′. It may be written in a general form as

$$Y_i = BA_i' + z \tag{III}$$

$$z = (y_i - \bar{y}) \cdot (1 - b) \tag{IV}$$

where Y_i is the biological age of an individual according to the concept; z is the correction; BA_i is the biological age of an individual calculated with the multiple regression method; y_i is the chronological age of an individual; $\bar{y}$ is the chronological age average for the whole sample (the regression center); b is the coefficient of simple linear regression which expresses the relation between the biological and chronological ages. It is important to note

$$b = R \cdot \frac{\sigma_{BA}}{\sigma_y}$$

that where R is the coefficient of multiple correlation which expresses the relation between the age and all the measured parameters in the population under study.

The sign and value of the correction

If an individual is at chronological age in the older part of the population ($y_i > \bar{y}$), it follows from expression (IV) that $z > 0$. In this case the correction is to be added to the underestimated BA values. Reversely, when $y_i < \bar{y}$ (the individual is in the younger part of the population), $z < 0$, the correction is to be subtracted from the overestimated BA value.

As follows from (IV), an absolute value of z depends on the magnitude of two factors, one expressing the distance of the individual's chronological age from the regression center, the other showing agreement of the regression considered with the linear model. The larger the distance of y_i from the regression center, the greater is an absolute value of the factor $(y_i - \bar{y})$, consequently, the greater is z. When $y_i = \bar{y}$, z is zero. Thus, when an individual is at chronological age near the regression center, that is in the region of the most accurate BA estimates, z is close to zero.

The role of the second factor in expression (IV) is associated with the fact that b can vary between 0 and 1. At low b which is the case at a moderate coefficient of multiple correlation between the measured parameters and the age, the factor $(1 - b)$ approaches 1 (its maximum). At high b (a large coefficient of multiple correlation), the factor as well as the correction z tend to zero.

Thus, the value of z increases the larger the distance of an individual's chronological age from the regression center and the less agreement of the regression with a linear model, that is the larger the deviation of the calculated BA from the theoretical values. Consequently, the correction z eliminates systematic errors inherent to the multiple regression model of biological age.

Calculation of BA with the correction z in cross-sectional studies

In the right-hand columns of Table 3 one can see biological age means for groups of individuals at the same age, calculated from the multiple regression equation but with the correction z. As can be seen from comparison with the left-hand column of the table, in this case the means are much closer to the chronological age than they are without the correction. In some of the groups (women of 93–94 years, men of 65 and 78 years) the difference between the corrected mean biological age and the chronological age exceeds one year, being, however, statistically insignificant.

Calculation of BA with the correction z in longitudinal studies

Typical examples which demonstrate how an individual's BA (calculated with the correction) changes in longitudinal studies are given in Figure 3 as plots of BA versus chronological age. BA of some individuals is behind their chronological age during the whole observation period (E, 4–5-years' delay; A, 8- to 9-years' delay), for the others the two values coincide (B,G). Plot D demonstrates that BA is 7 years ahead of the chronological age. In all these cases the rate of BA change was almost the same and approximately

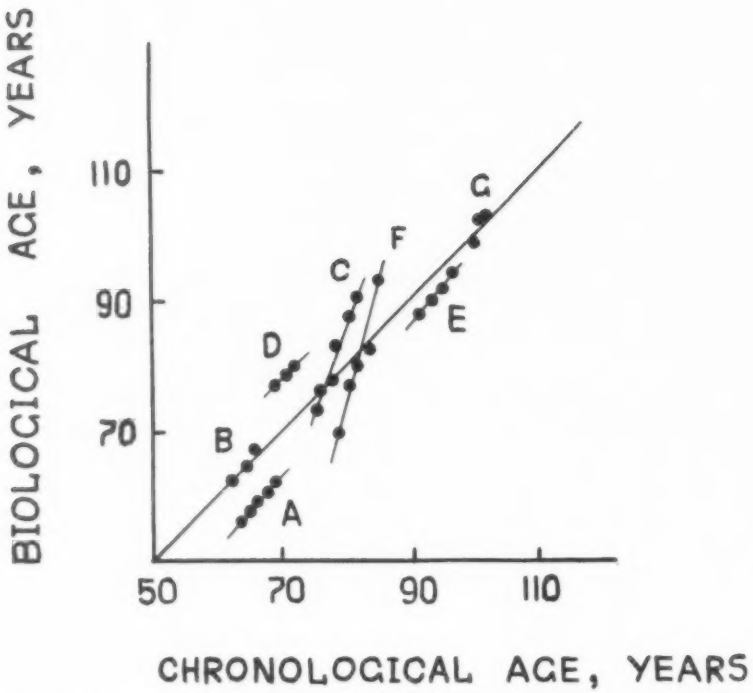

FIG. 3. Typical changes of individual biological age in longitudinal studies. A – men, examined at age 64–69 yrs; B – women (62–66 yrs); C – men (76–82 yrs); D – men (69–72 yrs); E – men (92–97 yrs); F – men (79–85 yrs); G – men (100–102 yrs). The values of biological age were calculated with equations (I) for women and (II) for men using the correction suggested.

equal to one year increase of the biological age during one calendar year ("the normal aging rate").

Plots C and F in Figure 3 demonstrate a different mode of aging when the rates of BA change are higher than the "normal" rate and vary from two to seven years of biological age per one calendar year. In the examples considered BA of the individuals who were biologically "young" at the moment of the first examination (C, BA is 4 years younger than the chronological age; F, it is 10 years younger) becomes equal to the chronological age in the subsequent years, then BA becomes more or less ahead of the chronological age.

The preliminary analysis has shown that the mode of aging of all men and women examined regarding the three indices coincides mainly with one of the four types described above. Though the parameters measured occasionally changed in the subsequent examinations following the inverse mode instead of the main trend, we have not found quite a long (more than of two years) stay of BA at the same or considerably lower level.

DISCUSSION

The main aim of this work is to substantiate introduction of a correction to the multiple regression method used for calculation of biological age and to demonstrate that the cor-

rected BA estimates may be of help in longitudinal studies. Therefore the problem on applicability and sufficiency of the physiological indices used is outside the scope of this work. It should however be mentioned that two parameters, vibrational sensitivity and hand grip strength, have been used for estimation of human biological age with the multiple regression (Heikkinen *et al.*, 1974; Furukawa *et al.*, 1975) or profile (Borkan and Norris, 1980) methods. The third parameter, a short-time memory, was also recommended for the test battery (Shock, 1978; Siegler *et al.*, 1982).

The subjects examined in this work are at the age from 60 to 100 years. There is no doubt that to make an operative "normal aging standard," that is to derive equations which describe biological age of the persons at all the ages in the population considered, it is necessary that the lower age extremum would begin since 20–30 years. However, for comparative estimation of the two methods used (the ordinary procedure and with the correction) and also for comparison of the changes of biological age of the same individuals in the course of longitudinal observations, such an age range, "cut" at the lower extremum, can be assumed reliable.

The following concept was the basis for judgment on the accuracy of the calculated individual biological age (Dubina *et al.*, 1983) and for derivation of the correction suggested here for improvement of the estimate accuracy: in a population of apparently normal individuals biological age of each group of persons at the same age is equal to their chronological age. Among people of 50, some look like 40-year-old persons, some like 60-year-old; the majority, however, suit their age. It follows from the very concept of biological age that mean biological age of all the persons of 50 years old is just 50 years. It is quite probable that the biological age of individuals at the same age distribution is consistent with the normal law.

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Since biological age of an individual is an integral value summing up the state of many systems and functions and is calculated on the basis of many or a few physiological parameters, the data on the distribution patterns of the indices studied could be useful to support correctness of the assumption adopted. The number of examinations carried out in this work is not sufficient to conclude on the peculiarities of the frequency distribution of the indices used among the individuals of the same chronological age. In the available literature we also have not found any data on the distribution pattern of vibrotactile sensitivity, hand grip strength or short-time memory. For the other parameters, however, particularly for the concentrations of 30 blood components, it has been demonstrated that among residents of Goetheborg at the age of 70 years, many of the indices are distributed following the normal law. Moreover, after abnormal persons had been rejected, the asymmetrically distributed indices were found to be decreasing in their asymmetry (Sten *et al.*, 1981).

The error in the individual BA assessed on the basis of the multiple regression model depends primarily upon one's chronological age or rather upon its distance from the middle age of the sample. Such an error which changes with the age is an obstacle for using this method in the longitudinal studies. The above said is especially true for individuals at the regression edges where the error is greatest. For gerontological studies both of animals and of man it is also essential that for the upper regression edge (old age) the multiple regression method gives underestimated values.

The correction suggested eliminates the effects of the individual's chronological age on the accuracy of the biological age assessment and allows estimation of the changes of

biological age of the same individual during repeated observations, which fact is of importance both in the studies of human aging and in gerontological experiments with animals.

Although this study is preliminary and rather serves to demonstrate the potentialities of the modification suggested for biological age assessment in longitudinal studies of aging, the data show that for people older than 60 years there are at least four types of the biological age changes. Three of them (BA is behind, ahead of the chronological age or coincides with it) have a normal aging rate. The fourth mode is characterized by a high aging rate. In accordance with Brown and Forbes (1976) the biological age "index" should reflect probability of the individual's death in the subsequent period of time. Studies of the relation between the mode of BA changes and the distance from the death will be a subject of our further investigations.

SUMMARY

Accuracy of individual biological age assessed using the physiological parameters on the basis of the multiple regression model depends upon the individual chronological age, the error of the assessment increasing with the distance from the median age in the sample. This characteristic is an obstacle for using the multiple regression method in the longitudinal studies. A correction is suggested which takes into account the distance of the individual's chronological age from the regression center and eliminates biological age estimate distortions inherent in the regression model.

By the example of apparently normal people of 60 to 100 years examined under the longitudinal program it has been shown that the biological age estimates obtained with the correction give better agreement between the mean biological age and the chronological one in a group of individuals at the same age as compared with the assessment made without the correction. In the studies, a hand grip strength, a short-time memory and a level of vibrotactile sensitivity were used as biological age indices. These indices have shown the following correlations with age: -0.736, -0.601 and 0.698 for women and -0.759, -0.548 and 0.591 for men, respectively. The multiple correlation coefficients of all the three indices with age were 0.836 for the women and 0.804 for the men.

The correction was used to compare the rates of the biological age change for different persons in the longitudinal studies. The preliminary analysis revealed that there are at least four modes of aging. The biological age of some persons changes for several years with a normal rate (≈ 1 year per year), being the same as the chronological age, behind or ahead of it. In particular cases a high rate was observed when the biological age increased by 2 to 7 years during one calendar year.

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AGE DEPENDENT CHANGES IN AVERSIVE RESPONSES OF MICE TO ETHANOL AND THEIR CORRELATION WITH LIFE DURATION

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Abstract—Age dependent changes in voluntary responses to an ethanol solution were observed in CF1 male control mice, and an experimental group provided 5% sucrose (w/v) as their only liquid for ten days beginning at the age of four weeks. This was a life duration study that began when the mice were ten weeks of age. An untreated group of C57/B1 mice was similarly observed. A 3% ethanol solution (v/v) was offered the CF1 mice, and for a comparable demonstration of aversion to ethanol in C57/B1 mice, an 18% ethanol solution was required. In all three groups, the curves of decreasing proportions of behaviorally aversive mice that occurs with advancing age closely parallels the survivorship curves.

In the CF1 experimentals a more rapid development of behavioral aversion during the early portion of the life cycle, and an earlier onset of its decline phase occurs. Mean life span was significantly shorter in this group than in controls.

The age dependent changes in another parameter, total fluid intake per gram body weight, were compared for short-lived and long-lived subgroups of the C57/B1 mice. This ratio is relatively high early in life, decreases to significantly lower levels in adulthood, and later increase with advancing age. In the short-lived contingent lowest values are attained around the age of 40 weeks compared to 100 weeks in long-lived mice; and the subsequent phase of increasing values is similarly shifted.

INTRODUCTION

AGE DEPENDENT changes in behavioral aversion to an ethanol solution were observed in prior studies of CF1 and C3H mice (Kahn, 1975). The proportions of subjects aversive to an ethanol solution offered in a free choice situation is relatively low early in life, increases to peak and plateau levels in adulthood, and subsequently decreases with advancing age.

The current studies were undertaken to determine whether there is an association between the decreasing intensity of behavioral aversion to ethanol and the increasing mortality observed with increasing age.

We have reported that provision of five percent sucrose (w/v) as the only liquid source

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during a sensitive period (around the age of four weeks) results in a more rapid development of behavioral aversion to ethanol (Kahn, 1981). This procedure was employed with the CF1 experimentals of this study in an attempt to produce alterations in the pattern of age dependent changes in behavioral aversion to ethanol, and to see whether parallel changes take place in mortality and life span. The C57/B1 mice were included to assess the intensity of behavioral aversion to ethanol in this relatively high ethanol consuming strain, and to see whether age dependent changes in this parameter parallel survivorship in this strain as well.

Ethanol consumption data for the CF1 controls were previously reported (Kahn, 1975). Data relating the decline in behavioral aversion to increasing mortality with increasing age, and the effect of an experimentally imposed condition on both parameters is newly reported here.

METHODS AND PROCEDURES

CF1 and C57/B1 male mice were obtained from Carworth Laboratories, caged individually, and provided Purina food blox and liquids *ad libitum*. The room temperature was kept at 72 ± 2°F, and a 12 hour light-dark cycle maintained.

There were 76 CF1 controls, and 20 experimentals pretreated with five percent sucrose (w/v) as their only liquid source for a period of ten days beginning at the age of four weeks. A three percent ethanol solution (v/v) was offered as the alternative to plain water in the CF1 consumption studies.

95 C57/B1 mice were studied. None were experimentally pretreated. To ensure use of a test solution of sufficient concentration to evoke aversive responses comparable to those of the CF1 mice offered three percent ethanol, randomly selected subgroups were provided different concentrations of ethanol. 20 mice were offered three percent ethanol (v/v), 36 six percent, 20 twelve percent, and 19 eighteen percent ethanol (v/v). The group provided the 18% solution demonstrated a range of age dependent changes in aversive responses similar to those demonstrated by CF1 mice offered three percent ethanol. The C57/B1 subgroups offered the lower concentrations of ethanol did not provide as sensitive a picture of age dependent changes. The data from the subgroup offered 18% ethanol were used to represent all 95 C57/B1 mice in this regard. Mortality data were closely similar for all four C57/B1 subgroups; therefore the mortality data derived from all 95 mice were used in relating this parameter to the ethanol response data of the subgroup offered 18% ethanol.

Consumption data for water and for the ethanol solution, offered at separate sites at the front of the cage, were obtained by weighing the bottles three times weekly for the first month of the study, and at weekly intervals thereafter. The two bottles were site interchanged after each observation period. Consumption of the ethanol solution at preferred and non-preferred sites was expressed as percent of total fluid intake.

Mice demonstrate "behavioral aversion" to ethanol when its consumption at preferred and non-preferred sites is less than 50% of total fluid intake. In the presence of aversive behavior, consumption of the ethanol solution when offered at the *preferred site* is the index of its intensity by inverse relationship (Kahn, 1969; 1972). "Highly aversive" behavior is demonstrated when the mean consumption of ethanol at the preferred site is less than 30% of total fluid intake.

The proportion of mice in a designated category of aversive behavior (as defined by its intensity) depicts the age dependent changes in this parameter. With CF1 mice much of the age dependent changes appear as movement into or out of the "highly aversive" category. These changes would be missed if the inclusive category of "behavioral aversion" was used as the index of such changes. With C57/B1 mice, even the subgroup provided 18% ethanol as test solution was not as intensely aversive as the CF1 groups offered 3% ethanol. Too few mice demonstrated "highly aversive" behavior to adequately portray the changes taking place in aversive behavior during developmental and aging phases. For these reasons the CF1 groups are reported as the proportions of "highly aversives"; while for the C57/B1 group the inclusive category of "behavioral aversion" is used.

Observations were begun with the CF1 mice when they were ten weeks of age, and with the C57/B1 mice at age 15 weeks, and were continued for their life duration. Body weights were obtained at intervals throughout the study.

Another parameter derived from the data employed in the C57/B1 study is the ratio of total fluid intake per gram body weight. This parameter is known to undergo its own characteristic pattern of age dependent changes (Kahn, 1977), and was used in this study as a basis for comparing long lived and short lived groups of C57/B1 mice. The chi-square, or Student *t* test, was used in appropriate situations for statistical analysis.

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RESULTS

The CF1 mice

The proportions of control and experimental mice highly aversive to three percent ethanol and of percent survivorship at different ages are presented in Figure 1.

Although the proportions of highly aversive experimentals are somewhat higher than in controls during the developmental phase, the differences were not statistically significant. A parallelism between the declining proportions of highly aversive mice and the declining proportions of surviving mice is evident in both groups. The phase of rapid decline in the proportions of highly aversive subjects begins about the age of 60 weeks in experimentals, but not until the age of 100 weeks in controls. The mean life span ±S.D. of 20 experimentals was 571 ± 160 days; that of 76 controls, 664 ± 150 days. The difference is statistically significant by the Student t test, $p < 0.025$.

Statistical significance of the decreasing proportions of highly aversive mice is difficult to evaluate when associated with an ongoing mortality that is substantial. However, the reality of a decreasing intensity of behavioral aversion to ethanol, as an aging phenomenon, can be confirmed in the observed behaviors of individual mice. 23 of the 69 control mice (33%) that died after the age of 60 weeks *had* demonstrated highly aversive behavior, succeeded by a period of *non-aversive* behavior prior to death. The remaining 46 mice were still highly aversive when they died. The transition to non-aversive behavior was completed (in those subjects demonstrating this change) at a mean age ±S.D. of 609 ± 146 days. Nine of the 16 experimentals (56%) that died after the age of 60 weeks demonstrated this transition to non-aversive behavior at a mean age of 497 ± 70 days. This is significantly earlier than in controls, $p < 0.01$ (Student t test).

Thus both control and experimental groups display a parallelism between the decline in proportions of highly aversives and the decline in proportions of survivors. The experimentals show a significantly earlier age of transition to non-aversive behavior and a significantly shorter mean life span.

The C57/B1 mice

For the reasons stated in Methods, survivorship data for all mice were compared with the ethanol response data from the subgroup offered 18% ethanol. The data are presented in Figure 2. The peak level in proportions of aversive mice occurs at the age of 60 weeks when 17 of 18 mice (94%) are aversive. By the age of 100 weeks only four of the 16 mice remaining alive (25%) are aversive. As in the CF1 study, the phase of rapid decline in proportions of survivors parallels the decline in proportions of aversive mice (as in Figure 2). The mean life span ±S.D. of the 95 C57/B1 mice was 844 ± 113 days.

In the subgroup offered 18% ethanol, 18 died after the age of 60 weeks (when the peak level of aversive behavior was attained). 14 of these mice (78%) demonstrated a shift from aversive to non-aversive behavior at a mean age of 693 ± 171 days (compared to their mean life span ±S.D. of 844 ± 113 days). Three mice remained aversive throughout the study, and one was non-aversive throughout.

The ratio of total fluid intake per gram body weight is a physiologic parameter known to undergo characteristic age dependent changes (Kahn, 1977). Long-lived and short-lived subgroups of the C57/B1 mice of this study were compared in this regard. There were 15

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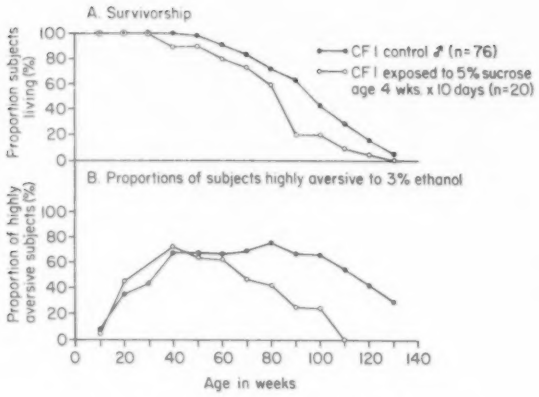

FIG. 1. **A.** Comparison of survivorship in control and experimental CF1 mice pretreated with 5% sucrose (w/v) as only liquid, at age 4 weeks for 10 days.
B. Comparison of proportions of highly aversive mice at successive ages in the same CF1 control and experimental groups.

mice with a life duration under 700 days (mean life span ±S.D. 610 ± 122 days), and 28 with a life duration over 900 days (mean life span 974 ± 43 days). Mean values for this ratio at successive ages are shown in Figure 3.

At the age of 15 weeks these values are closely similar in the two groups; 0.205 ± 0.026 cc/gm (N = 15) in the *short-lived* group, and 0.209 ± 0.031 cc/gm body weight (N = 28) in the *long-lived* group. From these levels significant decreases to low mean values occur in both groups. In the short-lived group a low of 0.133 ± 0.017 cc/gm (N = 14) is attained at the age of 35 weeks, a value significantly lower than that at age 15 weeks, $p < 0.001$ (Student *t* test). In the long lived group a low of 0.132 ± 0.021 cc/gm body weight (N = 27) is attained at the age of 95 weeks, a value significantly lower than at age 15 weeks, $p < 0.001$.

After the low values are attained, they increase significantly with age in both groups. In the long-lived group mean values increase to 0.150 ± 0.021 (N = 27) at the age of 115 weeks, $p < 0.001$ (Student *t* test). They were higher still at the age of 140 weeks (0.233 ± 0.033 cc/gm, N = 9), but the analysis of statistical validity of the difference to the low value at age 95 weeks is rendered difficult by the large number of deaths occurring by the age of 140 weeks.

In the short-lived group the values increase significantly from their low at age 35 weeks, to a peak of 0.207 ± 0.108 cc/gm (N = 11) at the age of 90 weeks ($p < 0.001$). To make sure that the three deaths occurring between these two data points have not distorted the probability calculation, the mean value for age 35 weeks was recalculated to include only those 11 subjects that survive until the age of 90 weeks. This made little difference in the mean, and no change in the statistical validity of the difference between these two values.

Mean values decrease more rapidly in the short lived group, and at the age of 35 weeks is significantly lower than that for the long lived group; 0.133 ± 0.017 (N = 14) versus 0.152 ± 0.018 cc/gm (N = 28) respectively, $p < 0.05$ (Student *t* test). Mean values con-

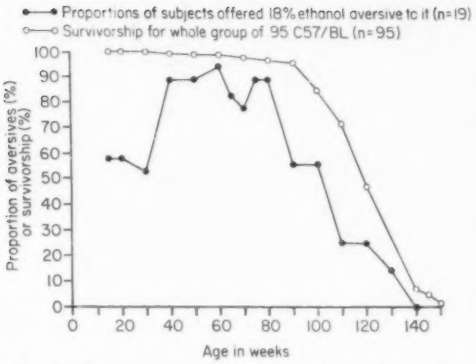
Proportions of subjects offered 18% ethanol aversive to it (n=19)
Survivorship for whole group of 95 C57/BL (n=95)
Proportion of aversives (%) or survivorship (%)
Age in weeks

FIG. 2. Progression of the proportions of aversive C57/B1 mice offered 18% ethanol (v/v) as alternative to plain water, compared to survivorship curve of whole population.

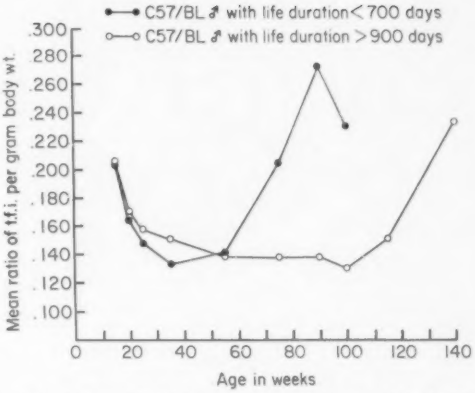

FIG. 3. Total fluid intake per gram body weight in 15 C57/B1 mice with life duration under 700 days, compared to 28 mice with life duration over 900 days.

tinue to decrease in the long lived group as they begin to rise in the short lived group. The two curves cross at a value of 0.140 cc/gm at the age of 55 weeks, as mean values in the long lived group continue to decrease to their low of 0.132 ± 0.022 (N = 28) at the age of 95 weeks. The rising values in the short lived group attain a peak at the age of 90 weeks, just prior to the low point attained by the long lived group. At the age of 90 weeks the mean is significantly higher in the short lived group than in the long lived group; 0.272 ± 0.125 (N = 11) versus 0.135 ± 0.016 cc/gm (N = 28) respectively, $p < 0.05$.

Thus there is a more rapid progression of these age dependent changes in the short lived group than in the long lived group during the early phase of the life cycle. The onset of the phase of increasing mean values begins earlier in the short lived group than in the long lived group; 35 weeks versus 95 weeks of age, respectively. The generally more rapid progression of age dependent changes in the short lived group is apparent in Figure 3.

DISCUSSION

The characteristic sequence of responses to the ethanol solution observed by us in prior studies (Kahn, 1975) was confirmed in this study. It includes an early phase with relatively low proportions of aversive (or highly aversive) mice, increasing to peak and plateau proportions in adulthood, and subsequently decreasing with advancing age. The full sequence of these responses was observed in many, but not all mice. Somewhat more than one third of the control CF1 mice, and somewhat over half of the experimentals demonstrated the shift from aversive to non-aversive behavior prior to death, while 78% of the C57/B1 mice did so. The differences between CF1 and C57/B1 groups in this regard may be attributable to differences in the intensity of the aversive response. Perhaps a more sensitive index for depicting changes in aversive behavior would have demonstrated some decline in those CF1 mice that did not demonstrate the transition to non-aversive behavior.

The basic findings of this study can be stated as follows:

1) The decline in proportions of mice behaviorally aversive to ethanol that occurs with

advancing age parallels the decline in the proportions of surviving mice in all groups studied. A decrease in the intensity of behavioral aversion to ethanol as an aging phenomenon was confirmed in the behavior of individual mice.

2) An experimentally imposed condition that resulted in an earlier mean age of transition from aversive to non-aversive behavior was associated with a reduction in mean life span.

3) Short-lived C57/B1 mice demonstrated a more rapid pace of age dependent changes in a physiologic parameter, total fluid intake per gram body weight, than do long-lived mice.

In regard to the first of these findings, a number of investigators have demonstrated how a decreasing competence of functional processes with age can determine the mortality curve of a population (Sacher, 1956; Strehler and Mildvan, 1960). Although behavioral aversion to ethanol cannot be considered a vital function *per se,* its decline does parallel the mortality curve. The possibility that the determinants of behavioral aversion are in some way related to vital functions should be considered. As a behavioral entity it must be mediated thru the CNS. The brain is a vital organ by virtue of its regulation and coordination of a host of physiologic functions (Hess, 1954; Konorski, 1967). As the brain ages these vital functions can be expected to decline in activity, as would non-vital functions mediated by the brain. Thus the decline in intensity of behavioral aversion to ethanol could be a reflection of generalized aging of the infrastructure of the CNS.

The second basic finding deals with the effects of providing five percent sucrose (w/v) as the only liquid source during a sensitive period around the age of four weeks. As to the mode of action of this pretreatment, we have observed that 1) the rate of development of aversive behavior to ethanol is *increased* when the five percent sucrose is provided at the age of four weeks, but not when offered at the age of 12 weeks (Kahn, 1981); 2) the rate of development of aversive behavior is *delayed* when pretreatment is provided during gestation (unpublished data); and 3) the effects of pretreatment provided during either of these sensitive periods on *other processes,* such as the development of fighting behavior, can be of *opposite sign* from that on the development of aversion to ethanol (unpublished data). For these reasons it appears unlikely that we are dealing with some general biochemical effect of five percent sucrose as provided, but with the reactions to this treatment by the regulatory mechanisms involved with age dependent activities of specific processes.

Alterations in the course of development evoked by conditions imposed during a sensitive period have been reported for many biologic processes (Levine and Lewis, 1963; Faris and Campbell, 1981; MacLusky and Naftolin, 1981). We have reported that such early pretreatment can affect later phases of the pattern of age dependent changes as well as the development phase (Kahn, 1968).

It is our hypothesis that the entire pattern of age dependent changes in activity of functional processes observed during a life cycle are predetermined by control mechanisms, alterable during a sensitive period early in life. Such a determinant is in fact demonstrated by the CF1 experimental group.

The third basic finding demonstrates a relationship between the rate at which age dependent changes proceed and life duration. Age dependent changes in the parameter, total fluid intake per gram body weight, proceeds more rapidly in the short-lived than in the long-lived group (Figure 3). A rate factor also appears to be in effect in the CF1 group experimentally pretreated with five percent sucrose. There is a somewhat more rapid development of behavioral aversion to ethanol, and an earlier onset of the decline phase (Figure 1B), suggesting a more rapid rate of age dependent changes throughout the life cycle, and associated with a decrease in mean life span. A correlation between a relatively

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rapid rate of progression of age dependent changes and a relatively short life span, and vice versa, is both reasonable and in keeping with the data.

Goldschmidt (1938), among others, reported many instances where experimentally induced changes in rates of development are produced by sensitive period effects. Based on evidence that the life span in poikilotherms is inversely proportional to the ambient temperature, Pearl hypothesized that life duration depends upon the "rate of living" (Pearl, 1928). In mammalia, caloric restriction results in a retardation of growth and an increase in life duration (McCay *et al.*, 1935). Our studies indicate that factors other than temperature manipulation in poikilotherms, or growth retarding caloric restriction in mammalia, can affect the rate at which age dependent changes proceed in particular systems; with associated effects on life duration.

The kinds of changes produceable in the pattern of age dependent changes during a life cycle are in all likelihood not confined to simple alterations of the overall rate of change. We have found that hemoglobin concentration develops more rapidly, and is maintained at higher plateau levels for a longer duration in mice exposed to partial asphyxiation during gestation, than in controls (Kahn, 1968).

It is apparent that much remains to be learned about the kinds of alterations in the pattern of age dependent changes that can be evoked by the early imposition of experimental conditions. Extension of our knowledge in this regard might well provide us with a comparable measure of control of life duration.

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SUMMARY

The expression of behavioral aversion to ethanol in mice demonstrates a characteristic pattern of age dependent changes in which its intensity is relatively low early in life, increases to peak and plateau levels in adulthood, and subsequently decreases with advancing age. In CF1 control and experimental groups, and in a group of C57/B1 mice, a parallelism was apparent between the decreasing proportions of behaviorally aversive subjects and the decreasing proportions of surviving subjects during the late phase of the life cycle.

The experimental CF1 group was provided five percent sucrose (w/v) as the only liquid source for ten days beginning at the age of four weeks. This resulted in a somewhat more rapid development of behavioral aversion to ethanol, and an earlier onset of the decline phase of this parameter relative to controls.

Short lived (life duration <700 days) and long lived (life duration >900 days) subgroups of C57/B1 mice were compared in regard to age dependent changes in another physiologic parameter, total fluid intake per gram subject. Age dependent changes in this parameter proceeded at a more rapid rate in the short lived than in the long lived subjects.

The hypothesis that regulatory mechanisms, alterable during a sensitive period, determine the subsequent pattern of age dependent changes of biologic processes, was advanced and discussed. The relationship between a relatively rapid rate of the progression of age dependent changes and a relatively short life span, and vice versa, was noted and discussed.

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CIRCADIAN VARIATIONS IN THE MONOAMINE LEVELS AND MONOAMINE OXIDASE ACTIVITY IN DIFFERENT REGIONS OF THE RAT BRAIN AS A FUNCTION OF AGE

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INTRODUCTION

FACTORS LIKE species, strain, sex, age, seasons, time of day and environment can influence brain amine levels. Striking differences in amine levels as a function of age have been reported. Similarly, various authors have observed time-related variations of the monoamine levels in the animal brain. An increased norepinephrine (NE) level in the midbrain and caudate nucleus of rat during the dark period was reported, which was attributed to increased motor activity (Friedman and Walker, 1968). An ultradian rhythm was reported for NE and dopamine (DA) of the whole brain of the rat (Scheving *et al.*, 1968). NE level of the lateral ventricle of the rhesus monkey exhibits peak during the light hours and trough during the dark hours (Perlow *et al.*, 1978). High concentrations of NE were observed during the dark phase in the hypothalamus and cervical spinal cord of the cat (Reis and Wurtman, 1968), hypothalamus of the rat (Manshardt and Wurtman, 1968), brain stem, diencephalon and cerebral cortex of the hamster (Morgan *et al.*, 1974) and in the frontal cortex and hypothalamus of the rabbit (Eleftheriou, 1974).

The cerebral DA of the rat showed an ultradian rhythm with increased values in the second half of the light and first half of the dark period (Lemmer and Berger, 1978).

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TABLE 1. MONOAMINE LEVELS IN THE *CEREBRAL CORTEX* OF THE AGING BRAIN WITH REFERENCE TO TIME.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Amine*
21-d-old	0.11 ± 0.011	0.10 ± 0.016	0.13 ± 0.016	0.17 ± 0.012***	0.12 ± 0.016	0.10 ± 0.016	Norepinephrine
3-m-old	0.22 ± 0.019	0.19 ± 0.011	0.16 ± 0.011	0.16 ± 0.021	0.24 ± 0.025	0.26 ± 0.032***	
6-m-old	0.27 ± 0.031	0.21 ± 0.011	0.17 ± 0.011	0.16 ± 0.014	0.22 ± 0.019	0.28 ± 0.037***	
12-m-old	0.26 ± 0.041	0.22 ± 0.022	0.19 ± 0.030	0.17 ± 0.015	0.21 ± 0.030	0.29 ± 0.028***	
24-m-old	0.27 ± 0.025	0.20 ± 0.025	0.16 ± 0.019	0.16 ± 0.019	0.22 ± 0.016	0.29 ± 0.035***	
21-d-old	–	–	–	–	–	–	Dopamine
3-m-old	0.014 ± 0.002	0.007 ± 0.001	0.005 ± 0.001	0.006 ± 0.000	0.012 ± 0.001	0.018 ± 0.001***	
6-m-old	0.018 ± 0.003	0.009 ± 0.002	0.005 ± 0.001	0.010 ± 0.002	0.014 ± 0.002	0.019 ± 0.002***	
12-m-old	0.020 ± 0.004**	0.012 ± 0.002	0.009 ± 0.002	0.011 ± 0.003	0.016 ± 0.003	0.017 ± 0.003	
24-m-old	0.018 ± 0.002***	0.011 ± 0.001	0.010 ± 0.001	0.014 ± 0.002	0.014 ± 0.002	0.016 ± 0.001	
21-d-old	0.11 ± 0.019	0.12 ± 0.016	0.16 ± 0.011***	0.16 ± 0.011	0.14 ± 0.025	0.10 ± 0.016	Serotonin
3-m-old	0.22 ± 0.022	0.26 ± 0.028	0.36 ± 0.029***	0.34 ± 0.035	0.29 ± 0.015	0.21 ± 0.016	
6-m-old	0.24 ± 0.030	0.27 ± 0.025	0.38 ± 0.019***	0.37 ± 0.044	0.30 ± 0.025	0.24 ± 0.022	
12-m-old	0.21 ± 0.017	0.24 ± 0.028	0.33 ± 0.024	0.35 ± 0.032***	0.28 ± 0.036	0.23 ± 0.031	
24-m-old	0.24 ± 0.030	0.28 ± 0.022	0.36 ± 0.029	0.37 ± 0.042***	0.31 ± 0.058	0.20 ± 0.028	

Values are given as μg/g wet tissue. Values are means ± SD. (n = 4).

***Difference between maximum and minimum values significant at $p < 0.001$.

**Difference between maximum and minimum values significant at $p < 0.01$.

TABLE 2. MONOAMINE LEVELS IN THE *CEREBELLUM* OF THE AGING BRAIN WITH REFERENCE TO TIME.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Amine*
21-d-old	0.100 ± 0.014	0.070 ± 0.011	0.110 ± 0.016**	0.110 ± 0.019	0.090 ± 0.007	0.090 ± 0.007	Norepinephrine
3-m-old	0.150 ± 0.007	0.140 ± 0.025	0.110 ± 0.016	0.100 ± 0.016	0.160 ± 0.025**	0.160 ± 0.021	
6-m-old	0.170 ± 0.011	0.130 ± 0.024	0.120 ± 0.002	0.100 ± 0.013	0.170 ± 0.014	0.180 ± 0.025**	
12-m-old	0.200 ± 0.019	0.170 ± 0.022	0.140 ± 0.022	0.120 ± 0.011	0.180 ± 0.015	0.200 ± 0.021***	
24-m-old	0.190 ± 0.024	0.160 ± 0.016	0.130 ± 0.021	0.150 ± 0.011	0.180 ± 0.035	0.210 ± 0.019**	
21-d-old	–	–	–	–	–	–	Dopamine
3-m-old	0.006 ± 0.001	0.003 ± 0.000	–	0.002 ± 0.000	0.007 ± 0.001	0.007 ± 0.001	
6-m-old	0.007 ± 0.001	0.005 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.008 ± 0.003*	0.008 ± 0.002	
12-m-old	0.009 ± 0.001***	0.005 ± 0.001	0.005 ± 0.001	0.004 ± 0.000	0.007 ± 0.001	0.009 ± 0.001	
24-m-old	0.006 ± 0.001**	0.004 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	
21-d-old	0 060 ± 0.011	0.090 ± 0.011	0.130 ± 0.016***	0.120 ± 0.016	0.130 ± 0.016	0.070 ± 0.014	Serotonin
3-m-old	0.100 ± 0.011	0.160 ± 0.011	0.200 ± 0.011***	0.180 ± 0.026	0.180 ± 0.026	0.130 ± 0.021	
6-m-old	0.120 ± 0.013	0.190 ± 0.011	0.240 ± 0.039***	0.210 ± 0.011	0.200 ± 0.016	0.160 ± 0.007	
12-m-old	0.130 ± 0.021	0.180 ± 0.016	0.260 ± 0.026***	0.240 ± 0.025	0.190 ± 0.016	0.140 ± 0.017	
24-m-old	0.120 ± 0.018	0.190 ± 0.025	0.240 ± 0.017***	0.200 ± 0.013	0.200 ± 0.025	0.140 ± 0.019	

Values are given as $\mu g/g$ wet tissue. Values are means ± SD. (n = 4).

***Difference between maximum and minimum values significant at $p < 0.001$.

**Difference between maximum and minimum values significant at $p < 0.01$.

*Difference between maximum and minimum values significant at $p < 0.02$.

TABLE 3. MONOAMINE LEVELS IN THE *MEDULLA OBLONGATA* OF THE AGING BRAIN WITH REFERENCE TO TIME.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Amine*
21-d-old	0.140 ± 0.019	0.120 ± 0.011	0.170 ± 0.016	0.190 ± 0.011***	0.180 ± 0.014	0.110 ± 0.007	Norepinephrine
3-m-old	0.410 ± 0.054	0.380 ± 0.041	0.230 ± 0.025	0.220 ± 0.025	0.380 ± 0.032	0.460 ± 0.030***	
6-m-old	0.520 ± 0.041***	0.460 ± 0.056	0.320 ± 0.021	0.310 ± 0.022	0.420 ± 0.041	0.480 ± 0.030	
12-m-old	0.560 ± 0.037***	0.440 ± 0.045	0.400 ± 0.028	0.360 ± 0.038	0.480 ± 0.042	0.560 ± 0.036	
24-m-old	0.440 ± 0.037***	0.360 ± 0.030	0.310 ± 0.043	0.280 ± 0.028	0.350 ± 0.030	0.410 ± 0.021	
21-d-old	0.016 ± 0.002	0.011 ± 0.002	0.018 ± 0.002	0.022 ± 0.002***	0.020 ± 0.002	0.012 ± 0.002	Dopamine
3-m-old	0.036 ± 0.002***	0.029 ± 0.003	0.012 ± 0.002	0.012 ± 0.002	0.028 ± 0.003	0.033 ± 0.003	
6-m-old	0.041 ± 0.005	0.032 ± 0.003	0.025 ± 0.003	0.026 ± 0.004	0.031 ± 0.003	0.044 ± 0.003***	
12-m-old	0.040 ± 0.004***	0.034 ± 0.003	0.026 ± 0.004	0.024 ± 0.002	0.032 ± 0.003	0.038 ± 0.003	
24-m-old	0.026 ± 0.003	0.021 ± 0.003	0.018 ± 0.001	0.018 ± 0.002	0.022 ± 0.002	0.029 ± 0.002***	
21-d-old	0.300 ± 0.025	0.360 ± 0.034	0.410 ± 0.030	0.420 ± 0.031***	0.390 ± 0.025	0.270 ± 0.025	Serotonin
3-m-old	0.410 ± 0.021	0.520 ± 0.031	0.670 ± 0.050	0.700 ± 0.043***	0.620 ± 0.029	0.460 ± 0.030	
6-m-old	0.480 ± 0.036	0.560 ± 0.048	0.730 ± 0.035	0.780 ± 0.043***	0.660 ± 0.019	0.490 ± 0.041	
12-m-old	0.480 ± 0.032	0.540 ± 0.059	0.700 ± 0.050	0.740 ± 0.041***	0.610 ± 0.036	0.500 ± 0.022	
24-m-old	0.420 ± 0.034	0.480 ± 0.035	0.560 ± 0.038	0.610 ± 0.068**	0.520 ± 0.038	0.400 ± 0.034	

Values are given as μg/g wet tissue. Values are means ± SD. (n = 4).

***Difference between maximum and minimum values significant at $p < 0.001$.

**Difference between maximum and minimum values significant at $p < 0.01$.

TABLE 4. MONOAMINE LEVELS IN THE *HYPOTHALAMUS* OF THE AGING BRAIN WITH REFERENCE TO TIME.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Amine*
21-d-old	0.200 ± 0.022	0.170 ± 0.016	0.210 ± 0.016	0.230 ± 0.014	0.240 ± 0.016***	0.190 ± 0.007	Norepinephrine
3-m-old	0.580 ± 0.041***	0.500 ± 0.028	0.360 ± 0.039	0.380 ± 0.030	0.480 ± 0.043	0.540 ± 0.041	
6-m-old	0.590 ± 0.048***	0.520 ± 0.068	0.400 ± 0.025	0.360 ± 0.034	0.480 ± 0.023	0.570 ± 0.031	
12-m-old	0.570 ± 0.030	0.530 ± 0.061	0.460 ± 0.046	0.440 ± 0.032	0.500 ± 0.026	0.590 ± 0.056**	
24-m-old	0.420 ± 0.058	0.360 ± 0.030	0.300 ± 0.025	0.310 ± 0.029	0.370 ± 0.034	0.430 ± 0.041**	
21-d-old	0.170 ± 0.011	0.120 ± 0.016	0.210 ± 0.019***	0.180 ± 0.014	0.200 ± 0.021	0.130 ± 0.016	Dopamine
3-m-old	0.450 ± 0.028	0.400 ± 0.019	0.280 ± 0.028	0.220 ± 0.011	0.360 ± 0.030	0.480 ± 0.025***	
6-m-old	0.540 ± 0.049**	0.500 ± 0.035	0.380 ± 0.037	0.360 ± 0.041	0.480 ± 0.030	0.530 ± 0.041	
12-m-old	0.520 ± 0.029**	0.470 ± 0.030	0.380 ± 0.054	0.370 ± 0.048	0.460 ± 0.045	0.500 ± 0.025	
24-m-old	0.320 ± 0.029***	0.280 ± 0.025	0.200 ± 0.019	0.180 ± 0.019	0.240 ± 0.021	0.310 ± 0.026	
21-d-old	0.210 ± 0.016	0.250 ± 0.028	0.300 ± 0.030	0.310 ± 0.031**	0.270 ± 0.008	0.220 ± 0.016	Serotonin
3-m-old	0.520 ± 0.039	0.580 ± 0.026	0.670 ± 0.043	0.710 ± 0.016***	0.710 ± 0.019	0.550 ± 0.041	
6-m-old	0.560 ± 0.039	0.610 ± 0.038	0.660 ± 0.030	0.740 ± 0.039***	0.640 ± 0.043	0.530 ± 0.041	
12-m-old	0.600 ± 0.058	0.660 ± 0.046	0.700 ± 0.028	0.710 ± 0.030***	0.610 ± 0.038	0.550 ± 0.028	
24-m-old	0.620 ± 0.028	0.680 ± 0.031	0.690 ± 0.022	0.700 ± 0.019***	0.600 ± 0.046	0.570 ± 0.034	

Values are given as μg/g wet tissue. Values are means ± SD. (n = 4).

***Difference between maximum and minimum values significant at $p < 0.001$.

**Difference between maximum and minimum values significant at $p < 0.01$.

TABLE 5. MONOAMINE LEVELS IN THE *STRIATUM* OF THE AGING BRAIN WITH REFERENCE TO TIME.

Age group	*Time (clock hours)* 02:00	06:00	10:00	14:00	18:00	22:00	*Amine*
21-d-old	0.070 ± 0.007	0.080 ± 0.011	0.110 ± 0.011***	0.100 ± 0.011	0.090 ± 0.007	0.100 ± 0.007	Norepinephrine
3-m-old	0.230 ± 0.025	0.210 ± 0.022	0.180 ± 0.022	0.130 ± 0.019	0.240 ± 0.028	0.260 ± 0.025***	
6-m-old	0.280 ± 0.021	0.230 ± 0.021	0.170 ± 0.019	0.160 ± 0.016	0.240 ± 0.028	0.280 ± 0.029***	
12-m-old	0.310 ± 0.021	0.260 ± 0.024	0.260 ± 0.025	0.210 ± 0.044	0.280 ± 0.039	0.340 ± 0.044**	
24-m-old	0.280 ± 0.028N.S.	0.270 ± 0.041	0.240 ± 0.019	0.240 ± 0.034	0.260 ± 0.025	0.260 ± 0.031	
21-d-old	2.910 ± 0.254	2.360 ± 0.128	3.610 ± 0.365***	2.860 ± 0.318	3.060 ± 0.169	3.000 ± 0.091	Dopamine
3-m-old	8.730 ± 0.288***	7.470 ± 0.436	4.080 ± 0.137	4.000 ± 0.154	6.930 ± 0.340	8.290 ± 0.107	
6-m-old	8.640 ± 0.656***	7.870 ± 0.496	5.070 ± 0.156	5.720 ± 0.167	7.810 ± 0.293	8.210 ± 0.239	
12-m-old	7.920 ± 0.400	7.640 ± 0.288	5.810 ± 0.273	6.210 ± 0.192	7.840 ± 0.301	8.410 ± 0.216***	
24-m-old	6.210 ± 0.165	5.830 ± 0.166	5.010 ± 0.280	4.940 ± 0.277	5.690 ± 0.363	6.420 ± 0.265***	
21-d-old	0.190 ± 0.016	0.240 ± 0.035	0.290 ± 0.025***	0.290 ± 0.022	0.260 ± 0.028	0.160 ± 0.022	Serotonin
3-m-old	0.480 ± 0.037	0.520 ± 0.030	0.610 ± 0.011***	0.610 ± 0.035	0.590 ± 0.035	0.500 ± 0.025	
6-m-old	0.420 ± 0.019	0.510 ± 0.037	0.660 ± 0.039***	0.640 ± 0.046	0.600 ± 0.019	0.480 ± 0.040	
12-m-old	0.400 ± 0.049	0.530 ± 0.055	0.680 ± 0.024***	0.600 ± 0.021	0.560 ± 0.030	0.430 ± 0.037	
24-m-old	0.420 ± 0.045	0.500 ± 0.039	0.580 ± 0.039	0.600 ± 0.029***	0.520 ± 0.042	0.470 ± 0.028	

Values are given as μg/g wet tissue. Values are means ± SD. (n = 4).
***Difference between maximum and minimum values significant at $p < 0.001$.
**Difference between maximum and minimum values significant at $p < 0.01$.
N.S. – Not significant.

TABLE 6. MONOAMINE LEVELS IN THE *MIDBRAIN* OF THE AGING BRAIN WITH REFERENCE TO TIME.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Amine*
21-d-old	0.150 ± 0.011	0.130 ± 0.011	0.200 ± 0.016	0.170 ± 0.016	0.220 ± 0.021***	0.180 ± 0.016	Norepinephrine
3-m-old	0.470 ± 0.032	0.410 ± 0.030	0.320 ± 0.030	0.220 ± 0.021	0.480 ± 0.028	0.500 ± 0.028***	
6-m-old	0.510 ± 0.046	0.470 ± 0.029	0.380 ± 0.037	0.330 ± 0.021	0.460 ± 0.029	0.520 ± 0.043***	
12-m-old	0.540 ± 0.049	0.480 ± 0.037	0.360 ± 0.041	0.380 ± 0.028	0.460 ± 0.031	0.600 ± 0.056***	
24-m-old	0.420 ± 0.031***	0.360 ± 0.051	0.300 ± 0.024	0.310 ± 0.016	0.380 ± 0.018	0.410 ± 0.029	
21-d-old	0.110 ± 0.025	0.100 ± 0.021	0.080 ± 0.016	0.120 ± 0.016**	0.090 ± 0.013	0.100 ± 0.016	Dopamine
3-m-old	0.260 ± 0.021***	0.190 ± 0.028	0.120 ± 0.022	0.090 ± 0.007	0.210 ± 0.011	0.230 ± 0.025***	
6-m-old	0.310 ± 0.034***	0.270 ± 0.029	0.190 ± 0.015	0.180 ± 0.019	0.230 ± 0.038	0.290 ± 0.029	
12-m-old	0.320 ± 0.039	0.260 ± 0.046	0.210 ± 0.011	0.190 ± 0.011	0.240 ± 0.025	0.360 ± 0.034***	
24-m-old	0.270 ± 0.029	0.220 ± 0.022	0.190 ± 0.016	0.190 ± 0.019	0.240 ± 0.022	0.290 ± 0.065***	
21-d-old	0.210 ± 0.025	0.270 ± 0.025	0.320 ± 0.014	0.340 ± 0.034***	0.300 ± 0.011	0.240 ± 0.021	Serotonin
3-m-old	0.600 ± 0.028	0.600 ± 0.022	0.760 ± 0.045***	0.720 ± 0.034	0.690 ± 0.016	0.580 ± 0.041	
6-m-old	0.600 ± 0.031	0.640 ± 0.040	0.760 ± 0.043**	0.730 ± 0.035	0.620 ± 0.039	0.600 ± 0.038	
12-m-old	0.580 ± 0.039	0.610 ± 0.047	0.700 ± 0.025	0.740 ± 0.052**	0.590 ± 0.037	0.540 ± 0.045	
24-m-old	0.500 ± 0.030	0.540 ± 0.041	0.610 ± 0.022	0.620 ± 0.031***	0.560 ± 0.043	0.480 ± 0.021	

Values are given as $\mu g/g$ wet tissue. Values are means ± SD. (n = 4).

***Difference between maximum and minimum values significant at $p < 0.001$.

**Difference between maximum and minimum values significant at $p < 0.01$.

TABLE 7. MONOAMINE OXIDASE ACTIVITY AND ITS CIRCADIAN VARIATIONS IN DIFFERENT AGE GROUPS AND DIFFERENT REGIONS OF THE RAT BRAIN.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Region*
21-d-old	0.028 ± 0.004	0.016 ± 0.005	0.019 ± 0.002	0.041 ± 0.004***	0.024 ± 0.002	0.026 ± 0.002	Cerebral cortex
3-m-old	0.037 ± 0.004	0.042 ± 0.003***	0.027 ± 0.002	0.037 ± 0.006	0.016 ± 0.004	0.035 ± 0.004	
6-m-old	0.021 ± 0.003	0.043 ± 0.005***	0.040 ± 0.005	0.033 ± 0.004	0.028 ± 0.004	0.024 ± 0.003	
12-m-old	0.017 ± 0.002	0.048 ± 0.003***	0.034 ± 0.005	0.043 ± 0.006	0.030 ± 0.004	0.032 ± 0.004	
24-m-old	0.028 ± 0.003	0.060 ± 0.008***	0.038 ± 0.003	0.044 ± 0.005	0.042 ± 0.004	0.036 ± 0.006	
21-d-old	0.008 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.018 ± 0.002***	0.007 ± 0.001	0.008 ± 0.002	Cerebellum
3-m-old	0.024 ± 0.003	0.041 ± 0.002***	0.025 ± 0.003	0.010 ± 0.001	0.022 ± 0.003	0.022 ± 0.002	
6-m-old	0.019 ± 0.004	0.038 ± 0.005***	0.031 ± 0.005	0.029 ± 0.004	0.016 ± 0.002	0.015 ± 0.002	
12-m-old	0.011 ± 0.001	0.049 ± 0.004***	0.022 ± 0.003	0.015 ± 0.002	0.019 ± 0.003	0.024 ± 0.005	
24-m-old	0.015 ± 0.002	0.050 ± 0.006***	0.021 ± 0.002	0.190 ± 0.003	0.017 ± 0.003	0.024 ± 0.003	
21-d-old	0.032 ± 0.003	0.030 ± 0.003	0.021 ± 0.003***	0.040 ± 0.005***	0.017 ± 0.002	0.028 ± 0.002	Medulla oblongata
3-m-old	0.047 ± 0.006	0.070 ± 0.006***	0.018 ± 0.002	0.043 ± 0.005	0.042 ± 0.006	0.040 ± 0.005	
6-m-old	0.038 ± 0.004	0.065 ± 0.007***	0.055 ± 0.005	0.048 ± 0.005	0.024 ± 0.003	0.021 ± 0.002	
12-m-old	0.026 ± 0.002	0.072 ± 0.004***	0.043 ± 0.003	0.030 ± 0.004	0.062 ± 0.004	0.052 ± 0.005	
24-m-old	0.040 ± 0.005	0.087 ± 0.007***	0.030 ± 0.004	0.052 ± 0.004	0.062 ± 0.007	0.062 ± 0.007	

Rate of kynuramine disappearance/mg protein/hr at 37°C. Values are means ± SD. (n = 4).
***Difference between maximum and minimum values significant at $p < 0.001$.

TABLE 8. MONOAMINE OXIDASE ACTIVITY AND ITS CIRCADIAN VARIATIONS IN DIFFERENT AGE GROUPS AND DIFFERENT REGIONS OF THE RAT BRAIN.

	Time (clock hours)						
Age group	*02:00*	*06:00*	*10:00*	*14:00*	*18:00*	*22:00*	*Region*
21-d-old	0.051 ± 0.004	0.024 ± 0.004	0.046 ± 0.005	0.044 ± 0.006	0.033 ± 0.004	0.059 ± 0.005***	Hypothalamus
3-m-old	0.036 ± 0.003	0.062 ± 0.003***	0.042 ± 0.004	0.030 ± 0.006	0.037 ± 0.006	0.052 ± 0.005	
6-m-old	0.058 ± 0.005	0.098 ± 0.007***	0.076 ± 0.008	0.058 ± 0.007	0.034 ± 0.005	0.040 ± 0.005	
12-m-old	0.056 ± 0.007	0.120 ± 0.010***	0.055 ± 0.007	0.029 ± 0.005	0.070 ± 0.007	0.083 ± 0.009	
24-m-old	0.063 ± 0.008	0.120 ± 0.013***	0.074 ± 0.006	0.058 ± 0.007	0.081 ± 0.008	0.094 ± 0.008	
21-d-old	0.036 ± 0.004	0.034 ± 0.005	0.042 ± 0.004	0.022 ± 0.003	0.036 ± 0.005	0.070 ± 0.008***	Striatum
3-m-old	0.056 ± 0.006	0.083 ± 0.008***	0.056 ± 0.007	0.044 ± 0.006	0.030 ± 0.004	0.075 ± 0.009	
6-m-old	0.041 ± 0.006	0.092 ± 0.009***	0.075 ± 0.004	0.071 ± 0.003	0.061 ± 0.007	0.033 ± 0.004	
12-m-old	0.048 ± 0.004	0.096 ± 0.010***	0.070 ± 0.009	0.045 ± 0.008	0.072 ± 0.008	0.068 ± 0.009	
24-m-old	0.050 ± 0.007	0.102 ± 0.018**	0.078 ± 0.009	0.048 ± 0.006	0.076 ± 0.008	0.074 ± 0.007	
21-d-old	0.044 ± 0.005	0.026 ± 0.005	0.041 ± 0.006	0.026 ± 0.004	0.018 ± 0.003	0.053 ± 0.007***	Midbrain
3-m-old	0.042 ± 0.006	0.071 ± 0.009***	0.053 ± 0.008	0.046 ± 0.007	0.034 ± 0.005	0.055 ± 0.007	
6-m-old	0.052 ± 0.008	0.086 ± 0.011***	0.061 ± 0.009	0.053 ± 0.009	0.036 ± 0.007	0.035 ± 0.007	
12-m-old	0.040 ± 0.006	0.106 ± 0.017***	0.069 ± 0.008	0.064 ± 0.008	0.074 ± 0.009	0.086 ± 0.010	
24-m-old	0.044 ± 0.007	0.118 ± 0.013***	0.077 ± 0.009	0.066 ± 0.004	0.079 ± 0.009	0.084 ± 0.010	

Rate of disappearance of kynuramine/mg protein/hr at 37°C. Values are means ± SD. (n = 4).

***Difference between maximum and minimum values significant at $p < 0.001$.

**Difference between maximum and minimum values significant at $p < 0.01$.

There are reports on 5-hydroxytryptamine (5-HT) in the whole brain (Albrecht and Visscher, 1956) and in the cerebral cortex of mice (Morgan *et al.*, 1974), whole brain of hamsters (Matussex and Patshke, 1963) in the whole brain (Dixit and Buckley, 1967; Montanaro and Graziani, 1967; Scheving *et al.*,1968), in the caudate nucleus and midbrain (Friedman and Walkar, 1968) of rats. In all these studies, higher levels of 5-HT were detected in tissues during the light period of the 24 hour cycle. Quay (1968) observed that the frontal cortex, the hypothalamus and the lateral lower brain stem were the regions in which the 5-HT rhythms were most distinct or of high amplitude during the light period.

It was suggested that the circadian changes in monoamine levels may be the result of the circadian variations in the activity of monoamine oxidase (MAO), the enzyme associated with the major degradative pathways of monoamine metabolism (Sharman, 1973). No such circadian variations were observed in the whole brain of the rat (Hillier and Redfern, 1976), whereas a clear time and age-related changes in MAO activity was reported in the whole brain of the rat (Radha, 1978). It is well known that changes in monoamine metabolism as a function of age results in a variety of central nervous system disorders, and each disorder is associated with the alteration of a particular amine and even related to a particular region of the brain. Since there is little information on biogenic amines with reference to age, different regions of the brain and different times of the day, it would be significant to study the inter-relationships of these amines and related enzymes, especially the major catabolic enzyme MAO in different regions of the brain in different age groups at different times of the day, which could help in a better understanding of some of the CNS disorders like manic depression, parkinsonism and insomnia.

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MATERIALS AND METHODS

A colony of male albino rats, *Rattus norvegicus albinus* (Wistar strain) of various age groups (21 days, 3, 6, 12 and 24 months old) were maintained at 28 ± 2°C under 12 hours light and 12 hours dark conditions. A clock controlled switch turned the lights on at 06:00 hours and off at 18:00 hours. Three to four animals were kept in each cage with water and food at *ad libitum*. The animals were decapitated at 02:00, 06:00, 10:00, 14:00, 18:00 and 22:00 hours and the brains were removed quickly and chilled. Dissections were performed on an ice-cooled glass plate. The different regions like cerebral cortex, cerebellum, medulla oblongata, hypothalamus, striatum and midbrain were dissected and separated as described by Glowinski and Iversen (1966). The brain regions were washed in ice cold saline to remove the blood clots, blotted and then transferred to precooled beakers and weighed. The method used for the estimation of NE, DA and 5-HT was that of Shellenberger and Gordon (1971). Final recovery, estimated in each experiment by internal standards, averaged 85%. Extracts from each region were analyzed for NE, DA and 5-HT content in duplicate. The amount of each amine is expressed as μg/g wet weight of the tissues L-Noradrenalin (FlukaAG, Buchs SG, Switzerland), Dopamine (Merck-Schuchardt, Munchen) and Serotonin creatinine sulfate (Calbiochem, California) were used as standards.

MAO activity was assayed basically as described by Weissbach *et al.* (1960), using kynuramine dihydrobromide (Sigma) as substrate. Protein content of these samples were estimated according to Lowry *et al.* (1951). The activity of monoamine oxidase is expressed as the rate of disappearance of kynuramine/mg protein/hour at 37°C.

Results were expressed as the mean ± standard deviation, and the values of maximum and minimum level was compared by using Student's *t*-test. When p values were higher than 0.05, differences were not considered statistically significant.

RESULTS

Figure 1 shows the occurrence of peak and trough levels of monoamine and monoamine oxidase activity of the aging cerebral cortex of rat brain. The variations observed in NE level of 21 day old rats were quite different from those of the other age groups studied. Maximum NE level was recorded during the light phase, whereas in all other age groups this occurs during the dark phase. A highly significant NE peak occurs at 22:00 hours in the case of 3, 6, 12 and 24 month old rats ($p < 0.001$), whereas the NE peak is at 14:00

CEREBRAL CORTEX

+ PEAK
- TROUGH

AGE	TIME (CLOCK HOURS) 02⁰⁰	06⁰⁰	10⁰⁰	14⁰⁰	18⁰⁰	22⁰⁰	
21-d-old		-		+			
3-m-old			-			+	
6-m-old				-		+	NE
12-m-old				-		+	
24-m-old				-		+	
21-d-old							
3-m-old			-			+	
6-m-old			-			+	DA
12-m-old	+		-				
24-m-old	+		-				
21-d-old			+			-	
3-m-old			+			-	
6-m-old			+			-	5-HT
12-m-old	-			+			
24-m-old				+		-	
21-d-old		-		+			
3-m-old		+			-		
6-m-old	-	+					MAO
12-m-old	-	+					
24-m-old	-	+					

FIG. 1. Occurrence of the peak and trough levels of norepinephrine, dopamine, serotonin and monoamine oxidase activity in the cerebral cortex of rat of different age groups.

hours in 21 day old animals ($p < 0.001$). No DA could be detected in the cerebral cortex of 21 day old animals throughout 24 hour cycle. In all other age groups the peak for DA was observed during the dark phase at 22:00 hours in the case of three and six month old rats and it was at 02:00 hours in the case of 12 and 24 month old rats. In all the age groups studied, 5-HT of cerebral cortex exhibited its maximum level during the light phase. Though there was a significant 5-HT peak ($p < 0.001$) in all the age groups in case of the cerebral cortex, there was not much variation in the levels during the entire light period that is, from 10:00 to 18:00 hours, the level remained low during the remaining period. MAO activity showed the peak at 06:00 hours in all the age groups except 21 days, which has the peak at 14:00 hours.

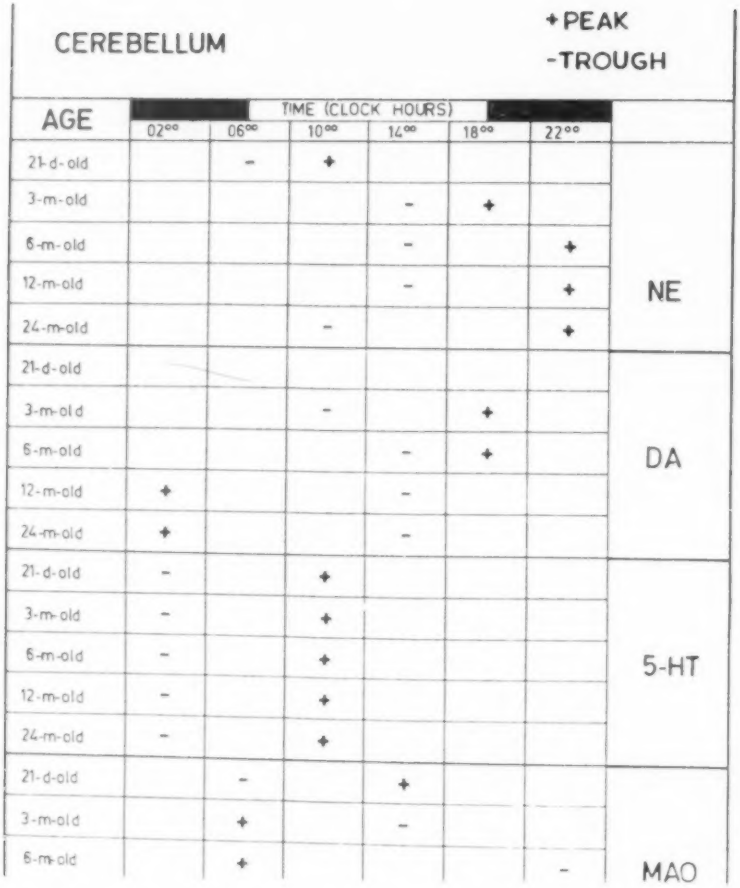

FIG. 2. Occurrence of the peak and trough levels of norepinephrine, dopamine, serotonin and monoamine oxidase activity in the cerebellum of rat of different age groups.

Figure 2 shows the appearance of the maximum and minimum levels of monoamines and MAO activity in the aging cerebellum. Except in 21 day old, in all other age groups, the maximum NE level was recorded during the dark phase of the L:D cycle. 21 day old rats has its maximum peak NE level during the light phase. No DA could be detected in the cerebellum of 21 day old, whereas in all other age groups though the peak was seen during the dark phase not much DA was found. Similar to cerebral cortex, 5-HT level in the cerebellum also shows the maximum level during the light phase with a significant peak at 10:00 hours and the trough at 02:00 hours in all the age groups. MAO activity follows the same pattern of cerebral cortex.

In contrast to other age groups, the medulla oblongata of 21 day old rat has its maximum NE, DA and 5-HT levels during the light period, whereas in other age groups NE

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MEDULLA OBLONGATA

+ PEAK
- TROUGH

AGE	TIME (CLOCK HOURS) 02⁰⁰	06⁰⁰	10⁰⁰	14⁰⁰	18⁰⁰	22⁰⁰	
21-d-old				+		-	NE
3-m-old				-		+	
6-m-old	+			-			
12-m-old	+			-			
24-m-old	+			-			
21-d-old		-		+			DA
3-m-old	+		-				
6-m-old			-			+	
12-m-old	+			-			
24-m-old				-		+	
21-d-old				+		-	5-HT
3-m-old	-			+			
6-m-old	-			+			
12-m-old	-			+			
24-m-old				+		-	
21-d-old				+	-		MAO
3-m-old		+	-				
6-m-old		+				-	

FIG. 3. Occurrence of the peak and trough levels of norepinephrine, dopamine, serotonin and monoamine oxidase activity in the medulla oblongata of rat of different age groups.

and DA follow a similar trend by having their peak level during the dark phase, and for 5-HT it is during the light phase (14:00 hours) of the L:D cycle. Medullary MAO activity pattern remains same as in the case of cerebral cortex and cerebellum (Figure 3).

The time and age-related variations of monoamine levels in the hypothalamus of rat brain (Figure 4) showed that NE, DA and 5-HT of 21 day old rats were more or less similar with the peak during the light phase, whereas with the other age groups, there is synchrony in the case of all the three amines, with the peaks during the dark phase for NE and DA and in the light phase of 5-HT.

Striatal amine levels of all the age groups other than 21 day old, has a more or less similar pattern for NE, DA and 5-HT in comparison with other regions studied, whereas in 21 day old, its NE and DA peaks were at 10:00 hours (Figure 5).

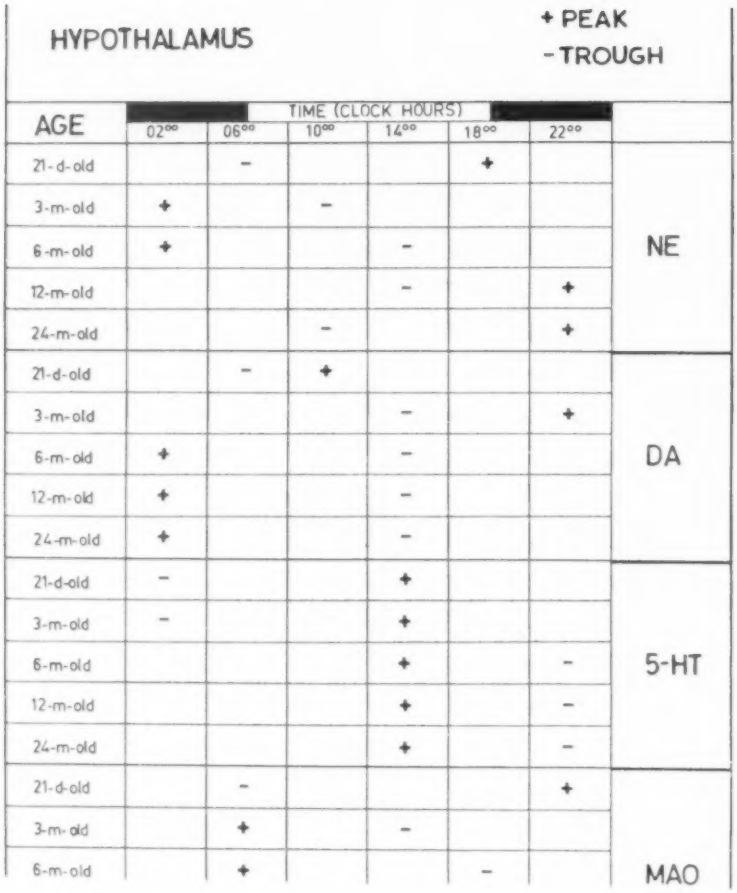

FIG. 4. Occurrence of the peak and trough levels of norepinephrine, dopamine, serotonin and monoamine oxidase activity in the hypothalamus of rat of different age groups.

Figure 6 shows the appearance of the maximum and minimum levels of aging rat midbrain. Similar to all other regions, midbrain follows a very similar trend in its rhythmic fluctuations of the monoamine levels in all the age groups other than 21 day old. Aging hypothalamus, striatum and midbrain of rat brain showed the maximum MAO activity at 22:00 hours in the case of 21 day old rats, whereas in other age groups the peak is at 06:00 hours.

DISCUSSION

The discrepancy observed in the time-related variations of monoamine levels and MAO activity of 21 day old rat brain regions when compared to other age groups, suggests the incomplete maturation of circadian rhythm. The report on the maturation of circadian rhythm of rat brain NE and 5-HT (Asano, 1971) showed that there are two circadian

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STRIATUM

+ PEAK
- TROUGH

AGE	TIME (CLOCK HOURS) 02⁰⁰	06⁰⁰	10⁰⁰	14⁰⁰	18⁰⁰	22⁰⁰	
21-d-old	-		+				NE
3-m-old				-		+	
6-m-old				-		+	
12-m-old				-		+	
24-m-old	+			-			
21-d-old		-	+				DA
3-m-old	+			-			
6-m-old	+		-				
12-m-old			-			+	
24-m-old				-		+	
21-d-old			+			-	5-HT
3-m-old	-		+				
6-m-old	-		+				
12-m-old	-		+				
24-m-old	-			+			
21-d-old				-		+	MAO
3-m-old		+			-		
6-m-old		+				-	
12-m-old		+		-			
24-m-old		+		-			

FIG. 5. Occurrence of the peak and trough levels of norepinephrine, dopamine, serotonin and monoamine oxidase activity in the striatum of rat of different age groups.

variations associated with the developmental stages of rat for both NE and 5-HT contents in the whole brain. For the period ranging from 8–9 to 15–17 post-natal days, these amines show a common circadian pattern, exhibiting an elevation in the dark and reduction in the light period and this pattern is altered on the 21st day. A new pattern of circadian rhythm is partly shaped on day 21 for 5-HT and on day 35–37 for NE and gets established on day 56–66 for both the amines. A consistent circadian pattern for the whole brain 5-HT content was reported in 35–37 day old rats (Okada, 1971). The present study is in agreement with that of Asano (1971). The present results have shown a well established

MIDBRAIN

+ PEAK
– TROUGH

AGE	TIME (CLOCK HOURS) 02⁰⁰	06⁰⁰	10⁰⁰	14⁰⁰	18⁰⁰	22⁰⁰	
21-d-old		–			+		NE
3-m-old				–		+	
6-m-old				–		+	
12-m-old			–			+	
24-m-old	+		–				
21-d-old			–	+			DA
3-m-old	+			–			
6-m-old	+			–			
12-m-old				–		+	
24-m-old				–		+	
21-d-old	–			+			5-HT
3-m-old			+			–	
6-m-old			+			–	
12-m-old				+		–	
24-m-old				+		–	
21-d-old					–	+	MAO
3-m-old		+			–		
6-m-old		+				–	
12-m-old	–	+					
24-m-old	–	+					

FIG. 6. Occurrence of the peak and trough levels of norepinephrine, dopamine, serotonin and monoamine oxidase activity in the midbrain of rat of different age groups.

peak for 5-HT in 21 day old rats, whereas both NE and DA did not show a well established peak on the 21st day. In immature rats (before weaning), it was observed that the rats tended to suck the breast of their mothers crouching in the corners of their cages during the light period. At the time of weaning they are more active; after 35–37 days the rats slept in the afternoon, the normal sleep time of the adult colony.

The results of this study show that DA level in all the brain regions of 3, 6, 12 and 24 month old rats have the highest level during the dark phase of the L:D cycle. The elevated levels of DA during the dark phase might reflect an increased role of DA in motor function during that period. Normally maintained animals show increased motor activity dur-

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ing the dark phase and are quiescent during the light phase. Striatal DA in particular play an important role in the regulation of motor activity (Owasoyo *et al.*, 1979). The peak level of NE in all the regions of all the age groups except 21 day old, occurred in the dark phase. It is, therefore, not surprising that the level of DA, a direct precursor of NE, also follows more or less a similar trend. 5-HT level in all the brain regions reached a peak at a time when the rats were inactive or asleep. Koella and Czicman (1966) associated 5-HT with sleep and postulated that 5-HT produces ocular signs of synchronized sleep by its action on receptor sites in the area postrema. Also, a correlation was found between the raphe system, the decrease in the cerebral 5-HT content and insomnia (Jouvet, 1969). A similar pattern for the daily rhythm in 5-HT content has been previously reported (Schriber and Schlesinger, 1971).

The present results have clearly indicated an age-related increase in MAO activity in different regions of the brain and its time-dependent variation. Further it was observed that the regional MAO activity rhythm in the case of 21 day old rats seems to be not fully synchronized showing the peak activity at 14:00 hours in cerebral cortex, cerebellum and medulla oblongata, whereas in other regions the peak occurs at 22:00 hours. A complete synchronization occurs at a later stage like three months and the peak occurs at 06:00 hours in all the regions, when all the amine levels are at their minimum.

As far as we know this is the first report of a complete profile of NE, DA, 5-HT levels and MAO activity and their interrelationship in different regions with reference to age and time of the day, which is very significant in understanding the age associated central nervous system disorders and the time-related information is very valuable for therapeutic purposes.

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SUMMARY

Time-related changes in the levels of norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT) and monoamine oxidase (EC 1.4.3.4, MAO) activity have been studied in the cerebral cortex, cerebellum, medulla oblongata, hypothalamus, striatum and midbrain of 21 day, 3, 6, 12 and 24 month old rats maintained at 12 hours of light and 12 hours of dark condition. Maximum NE level was seen during the dark phase in all the regions of 3, 6, 12 and 24 month old rats, whereas in 21 day old, the maximum NE level occurred during the light phase. In the cerebral cortex and in the cerebellum of 21 day old rat DA was absent at all times. In all the other age groups, the maximum DA level was seen during the dark phase, while for 5-HT higher level was during the light phase in all the age groups. MAO activity of 3, 6, 12 and 24 month old rats showed the peak activity at the beginning of the light phase (06:00 hours), whereas cerebral cortex, cerebellum and medulla oblongata of 21 day old rat had its peak MAO activity at 14:00 hours and at 22:00 hours in other regions.

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CENTROPHENOXINE INCREASES THE RATES OF TOTAL AND mRNA SYNTHESIS IN THE BRAIN CORTEX OF OLD RATS: AN EXPLANATION OF ITS ACTION IN TERMS OF THE MEMBRANE HYPOTHESIS OF AGING

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Abstract—The rates of total and polyA$^+$RNA (mRNA) synthesis were measured by radioisotope technique in the brain cortex of female CFY rats. There was practically no significant difference between the young (1.5 months) and adult (13 months) rats; however, the old group (26 months) displayed a considerable decrease of the rates of synthesis of both classes of RNA studied. Centrophenoxine treatment (100 mg per kg body weight per day, for 2 months) reversed this tendency, and increased significantly the synthesis rates of old rats almost to the adult level. The results are interpreted in terms of the membrane hypothesis of aging, attributing a free-radical scavenger function of the dimethylamino-ethanol incorporated into the nerve cell membrane from the centrophenoxine.

INTRODUCTION

Centrophenoxine (CPH) is an ester of p-chlorphenoxyacetic acid (PCAA) and dimethylaminoethanol (DMAE) (Pfeiffer *et al.*, 1957; Thullier *et al.*, 1959). It is used in the human therapy as a "neuroenergeticum", since it stimulates the metabolism of the brain. In particular, glucose uptake, oxygen consumption and carbon dioxide production increase significantly during CPH treatment both *in vivo* and *in vitro* (Nickel *et al.*, 1963; Herrmann and Dittmann, 1971; Oeriu *et al.*, 1973). Beneficial therapeutic effects of CPH have been observed in various human disorders such as cerebral atrophy, brain injury, postapoplectic status, chronic alcoholism, barbiturate intoxication, etc. (Schmidt and Broicher, 1970; Vojtechovsky *et al.*, 1970; Herrschaft *et al.*, 1974; Kugler, 1977).

It has also been observed that prolonged administration of CPH to healthy old animals reduced significantly the lipofuscin content of the brain nerve cells and myocardium (Nandy and Bourne, 1966; Nandy, 1968; 1971, 1978a, 1978b; Chemnitius *et al.*, 1970; Meier and Glees, 1971; Bourne, 1973; Hasan *et al.*, 1974a, 1974b; Riga and Riga, 1974; Spoerri *et al.*, 1974; Spoerri and Glees, 1975; Glees and Hasan, 1976). It is of importance from the point of view of experimental gerontology that the medium life-span of the CPH-treated animals was considerably longer than that of the old controls, and moreover, the learning capacity of the old treated mice proved to be improved as compared to the un-

treated age-matched controls (Hochschild, 1971; 1973a, 1973b; Nandy, 1977, 1978a; Nandy and Lal, 1977).

At the same time, CPH reversed the age-dependent accumulation of potassium in the intracellular space of brain cortical cells in old rats (Zs.-Nagy *et al.*, 1979), and a number of electron microscopic morphometric parameters of the synaptic structures of the cerebellar glomerulus were also improved (Bertoni-Freddari and Giuli, 1980; Bertoni-Freddari *et al.*, 1982; Giuli *et al.*, 1980). CPH injected *in vivo* in old rats for four weeks significantly reduced the water-insoluble protein fraction in the brain cortex, and the total protein (and dry mass) content displayed also a decreasing tendency (Zs.-Nagy *et al.*, 1981; Lustyik *et al.*, 1981). CPH stimulates the incorporation of ^{32}P into glycerophosphatides of isolated neurons and glial cells (Woelk, 1982), and decreases the "microviscosity" of the lipid layer in the synaptosomal fractions of the old rat brain *in vivo* (Nagy *et al.*, 1983). One of the latest publications states that glial cells of the human brain in a stationary culture system accumulate lipofuscin during the cultivation, but this accumulation can be decreased by a long-term treatment with CPH. This effect manifests itself in an increase of the rates of RNA and protein synthesis as well as of the glucose uptake (Ludwig-Festl *et al.*, 1983).

In spite of the relatively great number of available observations, the mechanism of action of CPH is still not quite clear. It is well established that the ester-bond of CPH is hydrolyzed in the organism. The biological effects of CPH are attributed to the DMAE, since the PCAA moiety is excreted very quickly by the urine. (Mitta *et al.*, 1967; Miyazaki *et al.*, 1971). Some authors claimed that DMAE enters the choline synthesis (Hochschild, 1973b; London and Coyle, 1978), and therefore, it is able to influence the synthesis of acetylcholine. Although this assumption has been discussed and contradicted by others (Bertoni-Freddari *et al.*, 1982), it may be true for the liver, but even in this case, it does not exclude the possible existence of some other metabolic pathways of DMAE in the brain, that is, behind the blood-brain barrier. As a matter of fact, it has been shown that DMAE becomes phosphorylated in the brain and persists for rather a long time in the membranes of nerve cells in form of phosphatidyl-DMAE (Miyazaki *et al.*, 1976). Since the DMAE proved to be a very efficient scavenger of hydroxyl free radicals *in vitro*, a mechanism of action of CPH was proposed based on the possible protective effect of DMAE in the membrane against free-radical induced cross-linking of the components (Zs.-Nagy and Nagy, 1980).

Our former experiments revealed a considerable age-dependent decline of the rate of total- and mRNA synthesis in the brain cortex of rats (Semsei *et al.*, 1982). These results and the known effects of CPH listed above suggested the necessity of the experiments described in the present paper. Furthermore, we intend to summarize our proposed mechanism of action of CPH in a more complete form, since the known effects of CPH can well be explained in terms of the membrane hypothesis of aging (Zs.-Nagy, 1978, 1979).

MATERIALS AND METHODS

All the experiments were carried out on female CFY white rats of our own breed. The terms young, adult and old indicate 1.5, 13 and 26 months of age, respectively. The rats were kept on the normal laboratory chow, water was available *ad libitum*.

Centrophenoxine treatment was carried out by intraperitoneal injection of 100 mg/kg body weight of Helfergin^{R} (PROMONTA, Hamburg, FRG) dissolved freshly in 0.5 ml physiological saline. CPH treatment started at the age of 24 months and was repeated daily for 60 days except Sundays. Control data regarding six

adult and seven old rats were taken from our previous paper comparing rats of the same breed and sex (Semsei *et al.*, 1982). The CPH-treated group consisted of three animals.

The reagents used as well as the method of preparation and measurement of total and mRNA synthesis rate were the same as described in our previous paper (Semsei *et al.*, 1982). Here we intend only to list the main steps of the procedure, in order to facilitate the understanding of the experimental set-up, without going into details.

1. In vivo pulse labeling. The rats were injected subcutaneously with 0.5 ml isotonic NaCl solution containing 2 μmole (5-^{3}H)-Uridine per 100 g body weight, and killed 45 minutes subsequent to this action by decapitation.
2. Samples of the brain cortex and the liver as well as the blood were taken as quickly as possible.
3. Samples were processed basically as described by Schmidt and Thannhauser (1945) using 4% perchloric acid for extraction of RNA. Distribution of the radiolabel was measured in the acid soluble, cytoplasmic fraction (and in the blood serum) and in the total RNA of the tissue. All the data were corrected for the pool size of the labeled precursor, in order to eliminate the effect of eventual differences in the availability of the precursor.
4. RNA extraction by the phenolic method. Total RNA was extracted by the phenolic method, and polyA$^+$RNA was separated by oligo(dT)-cellulose chromatography as described earlier (Semsei *et al.*, 1982). Rates of the RNA synthesis were calculated from the amounts of the incorporated radiolabel and the respective RNA contents of the tissues studied.
5. In order to eliminate any eventual differences in the experimental circumstances, reagent-solutions, labeled precursor, etc., young control rats were also processed during each measurement. The results of untreated and treated rats are expressed as percents of the respective young controls.

Statistical comparisons were carried out by using the Student's *t*-test. All further methodical details can be found in our previous paper (Semsei *et al.*, 1982).

RESULTS

We shall demonstrate the effect of CPH-treatment on the rates of RNA synthesis in relative units. We consider the average values obtained in young, simultaneously processed, untreated rats as 100%. The adult, old as well as old-CPH-treated, results will be related to this basis. It should also be noted that the results observed in adult (13 months) rats, although showed a slightly decreasing tendency, were statistically not different from those obtained in young rats except the mRNA synthesis rate when expressed in dpm/g dry weight units ($p < 0.05$) (Semsei *et al.*, 1982).

Figure 1 demonstrates the rate of total RNA synthesis as measured by the Schmidt-Thannhauser (1945) method in the liver and brain cortex. Significance values of the differences are indicated between the columns of the diagram. It is noteworthy that the total RNA synthesis rate increased very considerably in the CPH-treated group, and even the liver displayed an increase of this parameter under the effect of CPH.

Figure 2 shows the total and mRNA synthesis rates in the brain cortex as measured by using the phenolic extraction of RNA and subsequent separation of polyA$^+$RNA, as expressed in dpm/mg RNA units. Both parameters increased significantly under the CPH-treatment, however, the increase of mRNA synthesis rate is more considerable: it reached the untreated, adult level.

Figure 3 summarizes the rates of total and mRNA synthesis in the brain cortex as expressed in dpm/g wet or dry weight units, respectively. It is clear from the data that CPH exerts a considerable stimulating effect on the synthesis rates the extent of which depends somewhat on the way of expression, however, the tendencies are identical. The differences between the old and CPH-treated groups are statistically significant to such a high extent that there was no need to increase the number of animals in the CPH-treated group. CPH-treatment of two months reversed the age-dependent decline of the rates of total and mRNA synthesis and increased the figures obtained practically to the adult level.

It should be noted that in calculation of the synthesis rates on a dry weight basis, a rehydration of about 3% has been taken into account in the brain cell cytoplasm of the

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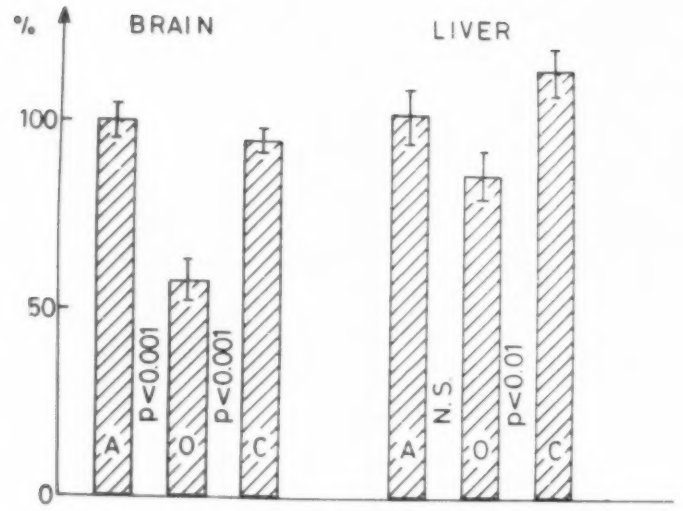

FIG. 1. The rates of total RNA synthesis in the brain cortex and liver of 6 adult (A), 7 old (O) and 3 CPH-treated old (C) rats as measured by the method of Schmidt and Thannhauser (1945) (mean ± S.E.M.). The results compared were expressed in cpm/g wet weight units originally. 100% represents the values obtained in untreated young rats processed parallel during each measurement. p stands for significance between the adjacent columns.

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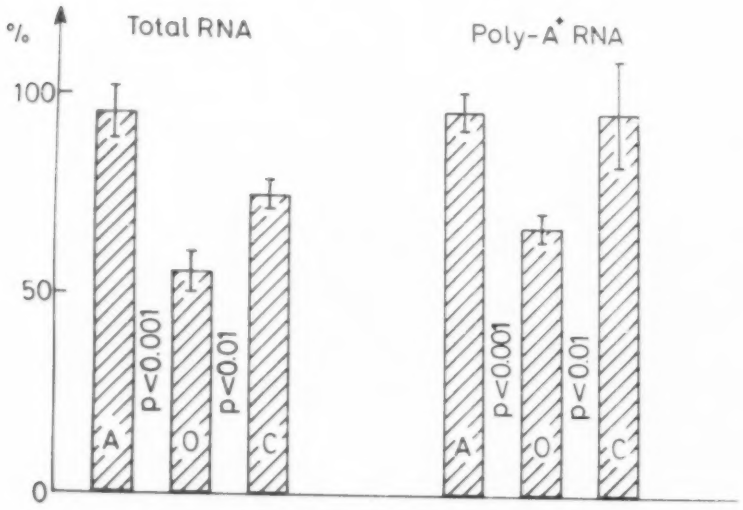

FIG. 2. The rates of total and polyA$^+$RNA synthesis in the brain cortex of 6 adult (A), 7 old (O) and 3 CPH-treated, old (C) rats, as measured by phenolic extraction and expressed in dpm/mg RNA units (mean ± S.E.M.). 100% represents the values obtained in young, untreated rats processed parallel during each measurement. p stands for significance between the adjacent columns.

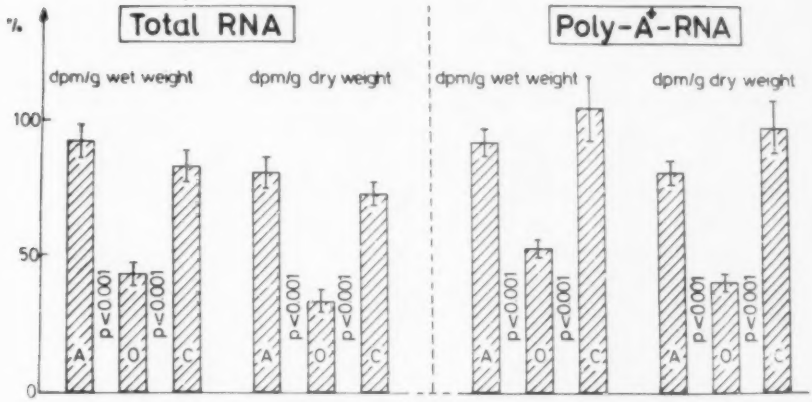

FIG. 3. The rates of total and polyA⁺RNA synthesis in the brain cortex of 6 adult (A), 7 old (O), and 3 CPH-treated, old (C) rats, as measured by phenolic extraction and expressed on wet and dry weight basis of the tissue (mean ± S.E.M.). 100% represents the values obtained in untreated, young rats processed parallel during each measurement. p stands for statistical significance between the adjacent columns.

CPH-treated group. This figure is justified by the experimental results of Lustyik *et al.*, (1981).

DISCUSSION

The method measuring RNA-content and the rates of total and polyA⁺RNA synthesis as described in our previous paper (Semsei *et al.*, 1982) and used here proved to be highly reproducible during the recent years in our laboratory. This method revealed a very considerable and consequent decrease in the rates of synthesis of both classes of RNA in the brain cortex of old rats, therefore, we can consider the data regarding age-dependence of this parameter as a safe basis of comparison.

There is no doubt that CPH-treatment is able to reverse the decrease of synthesis rates of both types of RNA when applied to old rats for two months. Since the increased rate of RNA synthesis allows the cell to perform the protein synthesis at a higher rate, it is logical to assume that the effect of CPH involves an increased rate of protein turnover as well. We are convinced that such a phenomenon must be related to all the beneficial anti-aging effects of CPH listed in the introduction of this paper.

The active mechanism of CPH was considered as being unclear even by one of the most recent papers (Ludwig-Festl *et al.*, 1983). Therefore, it seems to be necessary to summarize our proposed explanation for its action in terms of the membrane hypothesis of aging. This explanation, according to our knowledge, gives the most complete working hypothesis for further experiments with CPH or similar membrane-active drugs.

The membrane hypothesis of aging (Zs.-Nagy, 1978, 1979) attributes a primary role in aging to the decrease of passive potassium (and probably water) permeability of the membrane of postmitotic cells. The permeability losses may be due to free-radical induced cross linking as shown in various models (Zs.-Nagy and Nagy, 1980; Nagy *et al.*, 1983, etc.), involving both the lipids and proteins of the membrane. The decreased potassium permeability of the membrane causes an intracellular accumulation of potassium accom-

panied by a decrease of the intracellular water content (Pieri *et al.*, 1977; Zs.-Nagy *et al.*, 1978, 1979, 1982). From the point of view of cell excitability, the increase of intracellular potassium concentration is beneficial, since the cell membrane maintains its high resting potential and remains therefore excitable. Nevertheless, the increased intracellular potassium content results in an enhanced condensation of the chromatin and a consequent decline of the rates of RNA synthesis (Semsei *et al.*, 1982). This phenomenon may be the reason for the age-dependent decrease of gene expression, that is, a general decline of protein turnover (Richardson and Cheung, 1982).

The membrane hypothesis of aging as outlined above involves that the postmitotic cells gradually lose their ability to eliminate the damaged components of their body, that is, lipofuscin is accumulated in their cytoplasm.

It has been shown that DMAE is an active free-radical scavenger due to the relatively high electron content of its nitrogen atom (Zs.-Nagy and Nagy, 1980), and it is incorporated into the membrane of brain cells in form of phosphatidyl-DMAE (Miyazaki *et al.*, 1976). If the membrane components are relatively more protected against the cross-linking affect of free-radicals, the rate of their damage will be decreased, that is, their passive permeability for potassium (and probably for water) will relatively increase. Therefore, the intracellular potassium concentration decreases, the cytoplasm becomes rehydrated to a certain extent, and under such conditions the chromatin becomes more decondensed again, resulting in an increase of the rates of RNA synthesis, as shown by our present experiments. If this causes an increased turnover of the proteins, one can expect that the physiological properties of the cell membrane can further improve, and this cycle may seriously reduce the age-dependent damages of the nerve cells. In this assumption the lipofuscin content of the nerve cells is reduced by CPH primarily because the ability of the cells to eliminate these compounds is increased by the increased rate of protein synthesis.

Such an explanation is applicable to all observations concerning stimulatory effects of CPH (Nandy, 1978a, 1978b; Ludwig-Festl *et al.*, 1983, etc., see references in the introduction). Furthermore, some experimental proofs demonstrate that a chemically different drug like phytohemagglutinin (PHA) resembling the CPH only in the fact that it also increases the potassium permeability of the cell membrane, is also able to reverse several age-dependent impairments among others the decrease of RNA synthesis rate of rat brain *in vivo* by a similar ionic mechanism (Zs.-Nagy *et al.*, 1978; Semsei and Zs.-Nagy, 1983). On the other hand, it has also been shown that in rats deficient in vitamin E, the mechanism of the accelerated aging is due to the decrease of passive potassium permeability of the nerve cell membrane (Bertoni-Freddari *et al.*, 1981). One can draw the conclusion from these experimental results that crucial point of any anti-aging strategy of the cells is to maintain the permeability parameters of the cell membrane in the range where these parameters still do not exert any serious limiting effect on the synthetic machinery of the cells. CPH represents one of the artificial factors which may be useful for this purpose by *in vivo* application.

SUMMARY

Female CFY rats of two years received a daily dose of 100 mg of centrophenoxine (CPH) per kg body weight in form of intraperitoneal injection for two months. The rates of total and polyA$^+$RNA synthesis were measured by radioisotope techniques as follows:

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pulse labeling of 45 minutes was performed by using tritiated uridine. Total RNA was extracted from the brain cortex either by the method of Schmidt and Thannhauser (1945) or by the combined method with phenol-SDS-polyvinylsulphate. PolyA$^+$RNA was extracted subsequently by oligo-(dT)-cellulose chromatography. The results were compared to untreated adult (13 months) and old (26 months) control rats. Rates of synthesis were determined in the following units: cpm/g wet weight, dpm/mg RNA, dpm/g wet weight, dpm/g dry weight. Comparisons were made to the rates of synthesis observed in the brain cortex of young (1.5 months) rats processed parallel in each measurement, and the results are given as percents of the young values.

There is practically no significant difference between the young and adult rats, however, the old ones display a very considerable decrease of the synthesis rates of both classes of RNA studied. CPH treatment reversed this tendency, and increased significantly the rates of RNA synthesis of old rats almost to the adult level. The results agree with the general anti-aging effects of CPH and can be interpreted in terms of the membrane hypothesis of aging. This hypothesis attributes a free-radical scavenger function of the dimethylamino-ethanol incorporated into the nerve cell membrane from CPH.

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VITAMIN E EXTENDS LIFESPAN IN THE SHORT-LIVED ROTIFER *ASPLANCHNA BRIGHTWELLI*

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Abstract—Supplementation with vitamin E at a concentration of 25 μg/ml significantly increased the mean lifespan of the rotifer *Asplanchna brightwelli*. Of the three life stages, the prereproductive, reproductive and postreproductive, only the prereproductive stage was significantly extended by vitamin E supplementation. Vitamin E supplemented rotifers were larger than control rotifers in their early stages of life, but both groups attained the same size by the end of their lifespan.

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INTRODUCTION

VITAMIN E SUPPLEMENTATION increases the lifespan of a variety of invertebrate species: these include *Drosophila* (Miquel, Binnard and Howard, 1973), the rotifer *Philodina* (Enesco and Verdone-Smith, 1980) and several species of nematodes (Epstein and Gershon, 1972; Kahn and Enesco, 1981; Zucherman and Geist, 1983). These are all relatively short-lived species; 50 days for *Drosophila,* 19 days for *Philodina* and 45 days for the nematode *Turbatrix aceti.*

The objective of the present study was to determine whether vitamin E supplementation would influence the lifespan of an even shorter lived species. The rotifer *Asplanchna brightwelli* used in this study has a five day life-span. It is thus the shortest lived species in which the effect of vitamin E has ever been tested for its influence on lifespan or fecundity. The short lifespan of *A. brightwelli,* its ease of maintenance and the fact that its genetic uniformity is ensured by diploid parthenogenesis make it an interesting organism for aging studies. The lifespan of this rotifer can be influenced by temperature variations and by dietary restriction (Verdone-Smith and Enesco, 1982).

METHODS AND MATERIALS

Culture methods

Asplanchna brightwelli, clone 4B61 was originally obtained from Dr. John Gilbert, Dartmouth College, Hanover, N.H. The rotifers were fed *Paramecium caudatum* innoculated with *E. coli* in a Cerophyll medium as described in a previous paper (Enesco and Verdone-Smith, 1980).

Rotifers of known age were individually segregated into each of the wells of a 24-well tissue culture dish (No. 76-063-05) from Flow Laboratories, McLean, Virginia. When it was noted that the rotifer was about to give birth, it was transferred into control medium or into experimental medium containing vitamin E or solubilizing agent. Thus, the newborn rotifer was born into the appropriate medium. It would be exposed to this medium for 0 to 3 hours prior to birth and was then followed throughout its lifespan.

Each of the wells was checked every three hours to collect lifespan and fecundity data. One ml of medium was removed and replaced with fresh medium at each observation period. Transfer of the rotifers were carried out using a micropipette at a 5 μl setting (Finnipipette Ky, Helsinki, Finland). The rotifers were maintained at a temperature of 19.0°C. There were 24 rotifers in each experimental group.

Solubilization of vitamin E

Vitamin E, dl-α-tocopherol (lot 19C-0439, Sigma Chemical Company, St. Louis, Mo.) was solubilized in Tween 80 so it would dissolve in the aqueous culture medium, using the method described by Enesco and Verdone-Smith (1980). Alternatively, the vitamin E was solubilized in ethanol. A stock solution was prepared by dissolving the vitamin E in 99% ethanol with continuous stirring at 4°C. Appropriate dilutions were then added to the culture medium. Since vitamin E has an absorption maximum of 294 nm, its exact concentration in the rotifer medium could be verified from a standard curve. These measurements were taken using a Pye-Unicam scanning U.V. spectrophotometer.

Size measurements

Rotifers were both stained and immobilized by placing them in 0.1 mg/ml neutral red for 5 minutes. The length and width of the rotifers was measured at 96X magnification using a calibrated ocular micrometer on a Zeiss Ultraphot II Phase Contrast Microscope.

Statistical analysis

A one-way analysis of variance (ANOVA) followed by the post hoc Tukey test was carried out using the formula described by Brunning and Kinz (1977) and Sokal and Rohlf (1981).

RESULTS

In an initial series of experiments, various concentrations of vitamin E ranging from 5 to 100 μg/ml were solubilized in equal concentrations of the detergent Tween 80 and tested to determine their effect on rotifer lifespan. As a control similar concentrations of Tween 80 alone were also evaluated for their effect on lifespan.

The data obtained from this study, plotted in Figure 1, shows that Tween 80 alone had very little effect on rotifer life-span at any concentration. Figure 1 emphasizes that vitamin E caused a pronounced increase in life-span only at the concentration of 25 μg/ml. Statistical analysis confirmed these observations. A one way ANOVA followed by a *post hoc* Tukey test showed that there was a significant increase in lifespan only for those rotifers supplemented with 25 μg/ml vitamin E (alpha = 0.01). The solubilizing agent, Tween 80, had no effect on lifespan. Because of these findings, vitamin E was used at the concentration of 25 μg/ml in all subsequent experiments.

The oily vitamin E was solubilized in two different ways to make it available to the rotifers in their aqueous media: either by using the detergent Tween 80 or by dissolving it in the polar solvent ethanol. It should be noted that vitamin E alone without any solubilizing agent remains as an oily droplet on the surface of the medium and has no effect on rotifer lifespan. As shown in Table 1, vitamin E significantly extended the lifespan of the rotifers no matter which solubilization method was used (alpha = 0.01). Table 1 also shows that neither solubilizing agent had any detrimental effect on lifespan. There were no significant differences in lifespan between Tween control, ethanol control and untreated control rotifers.

For further analyses of vitamin E effects, the lifespan of the rotifers was divided into three periods based on fecundity data; they were the prereproductive, the reproductive and the postreproductive period. As shown in Table 1, vitamin E did not influence the length of the reproductive period or the number of offspring produced. Neither did it influence the length of the postreproductive period. In contrast, the length of the

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TABLE 1. THE EFFECT OF 25 μG/ML OF VITAMIN E DISSOLVED IN TWEEN 80 OR IN ETHANOL ON THE LIFE-PROFILE OF ROTIFERS IS SHOWN (N = 24)

	Untreated control	*Ethanol control (25 μl/ml)*	*Tween control (25 μg/ml)*	*Vitamin E/ Tween (25 μg/ml)*	*Vitamin E/ ethanol (25 μg/ml)*
Ave. Life-span ± S.E. (days)	5.46 ± 0.13	5.63 ± 0.17	5.29 ± 0.08	6.38 ± 0.12*	6.81 ± 0.10*
Ave. Prerep. ± S.E. (days)	2.31 ± 0.05	2.48 ± 0.06	2.46 ± 0.05	2.67 ± 0.06**	3.06 ± 0.09*
Ave. Reprod. ± S.E. (days)	3.00 ± 0.15	2.96 ± 0.14	2.73 ± 0.09	3.35 ± 0.14	3.50 ± 0.12
Ave. Postrep. ± S.E. (days)	0.14 ± 0.06	0.10 ± 0.04	0.10 ± 0.04	0.33 ± 0.08	0.19 ± 0.07
Ave. Offspring No./Rotifer	9	9	8	9	9

*significantly different from controls at alpha = 0.01
**significantly different from controls at alpha = 0.05

prereproductive period was significantly increased by vitamin E supplementation. It is this increase in the prereproductive period which is responsible for the overall increase in lifespan in the vitamin E treated groups.

Survival curves for all five experimental groups are shown in Figure 2. This presenta-

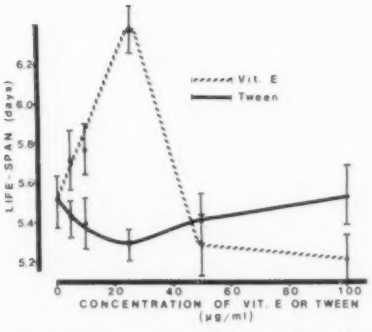

FIG. 1. The life-span of *A. brightwelli* in days is plotted against the concentration of vitamin E/Tween 80 and Tween 80 to which they were exposed (n = 24 at each concentration).

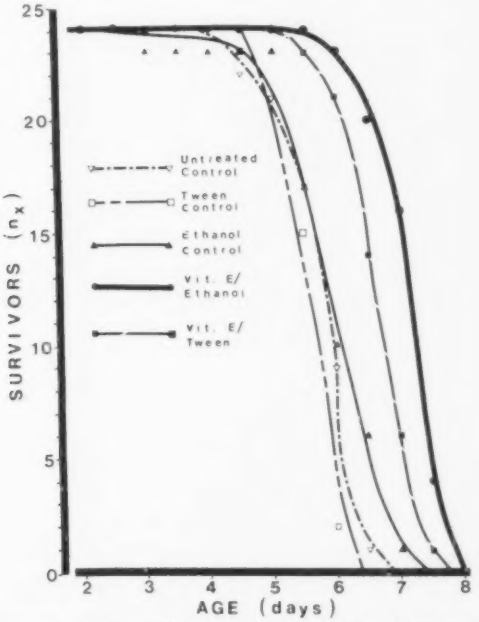

FIG. 2. Survivorship curves of *A. brightwelli* exposed to vitamin E solubilized in Tween 80 or in ethanol and controls are shown (n = 24).

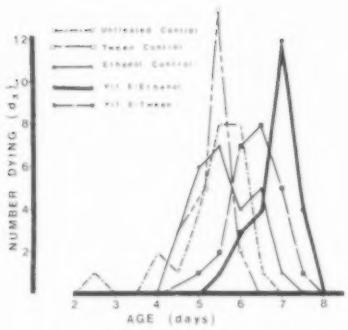

FIG. 3. The age specific death rates (dx) for *A. brightwelli* exposed to vitamin E solubolized in Tween 80 or in ethanol and controls are shown (n = 24).

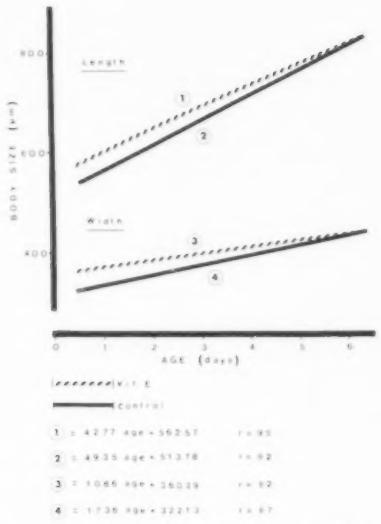

FIG. 4. The body length and body width of *A. brightwelli* exposed to 25 μg/ml of vitamin E is compared with that of untreated control (n = 24).

tion emphasizes that the curves for the two vitamin E treated groups are shifted to the right so that vitamin E is acting throughout the lifespan for all the rotifers. Vitamin E is not simply extending the lifespan of a few individuals. This point is further substantiated by an examination of Figure 3 which shows the age specific death rate for the five groups of rotifers. The age specific death curves shows that rotifers exposed to vitamin E dissolved in Tween or in ethanol had a peak death period later than the controls.

Since the vitamin E supplemented rotifers appeared to be larger than the control rotifers, size measurements of their length and width were taken. The results of these body size measurements are shown in Figure 4. Since the size measurements of all the control groups were virtually identical to each other, only the size measurements for the untreated control group are shown in Figure 4. While young vitamin E supplemented rotifers are clearly larger than untreated control rotifers, this size difference is gradually reduced with age. Thus both control and vitamin E supplemented rotifers attain the same limiting size by the end of their lifespan.

DISCUSSION

Our findings that vitamin E supplementation increases the mean lifespan of *A. brightwelli* by 17% is in agreement with other reports of the effects of vitamin E on lifespan. Vitamin E increased the lifespan of the longer-lived rotifer *Philodina* by 9% (Enesco and Verdone-Smith, 1980) that of the nematode *Turbatrix aceti* by 34% (Kahn and Enesco, 1981) that of the nematode *Caenorhadbitis elegans* by 23% (Epstein and Gershon, 1972). The increase in mean lifespan in the present study is due to an increase in the prereproductive phase of the lifespan.

In the present study, vitamin E had no effect on the length of the reproductive period or on the number of offspring produced. This is in contrast to observations on the longer lived

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rotifer *Philodina* (Enesco and Verdone-Smith, 1980) and on the nematode *Turbatrix aceti* (Kahn-Thomas and Enesco, 1982) where vitamin E supplementation increased both the length of the reproductive period and the number of offspring. The extremely short lifespan of *A. brightwelli* may account for this difference.

The results show that vitamin E significantly influenced lifespan only at the concentration of 25 μg/ml. At 5 and 10 μg/ml, vitamin E has a slight but insignificant effect on lifespan. The data plotted in Figure 1 suggest that these lower concentrations may represent points on a dose-response curve peaking at 25 μg/ml. The controls used in our experiments eliminate the possibility that the solubilizing agents used influence rotifer lifespan. The fact that vitamin E solubilized by two different means had a similar effect on rotifer lifespan eliminates the possibility that synergistic action between the vitamin E and the solubilizing agent could account for our results.

It is important to note that the size difference between vitamin E and control rotifers is greatest at the very beginning of their life, so that vitamin E must exert its influence on size within the first hours of life. Gilbert and Thompson (1968) has shown that vitamin E is responsible for the chain of events that transforms a rotifer population from an amictic or asexually reproducing to a mictic or sexually reproducing population. The size increase that we noted may be one of the first steps in this transition, even though sexually reproducing individuals would not be produced until the subsequent F_2 generation. Our data shows that the size difference is no longer present by the end of the lifespan.

The fact that vitamin E extends lifespan in a number of species is often interpreted in relation to the free radical theory of aging (Harman, 1968). However, an alternative explanation has been suggested by Fleming and Yengoyan (1981). These investigators report that vitamin E and other antioxidants which extend life-span in *Drosophila* act by depressing oxygen consumption. They conclude that life-span in *Drosophila* is primarily a function of metabolic rate. It may be that lifespan in rotifers is also primarily a function of metabolic rate. The lifespan of this rotifer is also extended by lowering the temperature (Verdone-Smith and Enesco, 1982). The possibility should be kept in mind that both vitamin E and cold treatment may extend lifespan by lowering the metabolism of the rotifers.

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ENHANCED ENDOCYTOSIS BY AGING CHONDROCYTES AND FIBROBLASTS

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INTRODUCTION

GERONTOLOGICAL STUDIES can be loosely divided into the causal and the pathological. Whereas the former seek the reasons for aging, the latter pertain more to the diseases of aging and the prospect of their possible amelioriation. In this perspective, we are examining the hypothesis that changes in the physiology of aging chondrocytes contribute to the production of primary osteoarthritis, a disease whose frequency increases markedly with age.

Although the chondrocyte is not normally considered a phagocytic cell, ultrastructural examination of cartilage has suggested the endocytosis of erthrocytes during haemarthrosis (Cotta, 1974) and collagen fibers during rheumatoid arthritis (Mitchell and Shepard, 1978). Furthermore, Canedy *et al.* (1983) have suggested that a "phagocytic phase" of chondrocytic activity occurs in the sequence of events leading to degenerative arthritis.

In the present study, we have measured the uptake of latex beads by cultured chondrocytes during aging *in vivo* and *in vitro*. To simplify interpretation of these experiments, early and late passage human fibroblasts have also been investigated.

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MATERIALS AND METHODS

Cell culture

Young (6 month old) and retired breeder (3 year old) rabbits were purchased from Hilltop Lab Animals, Scottdale, Pennsylvania. Young (3 month old) and old (2 year old) rats were purchased from Zivic-Miller Laboratories, Inc., Allison Park, Pennsylvania. Primary monolayer cultures of chondrocytes were established from the articular cartilage of these animals by the method of Green (1977), using Ham's F-12 medium supplemented with 10% (w/v) fetal bovine serum, 100 units/ml penicillin and 100μg/ml streptomycin. Cultures were incubated in 25cm² culture flasks with 4ml medium in an atmosphere of 95% air and 5% CO_2. When confluent, the cells were trypsinized and reseeded at a 1:2 or 1:4 split ratio. Monolayer cultures of young rabbit articular chondrocytes were allowed to senesce *in vitro*, as described by Evans and Georgescu (1983).

Human diploid lung fibroblasts (IMR90) were obtained from the N.I.A. aging cell repository at passages 6 and 16. They were cultured in Ham's F-12 medium supplemented with 20% fetal bovine serum and antibiotics. Confluent cultures were subcultured at a 1:2 split ratio.

Endocytosis

Polystyrene beads (Sigma, St. Louis, Missouri; 1.23μm diameter) were added to sub-confluent monolayer cultures to give a final concentration of 10^4 beads per cell. This concentration of beads was not rate-limiting for their internalization (data not shown). Duplicate cultures were incubated at 37° for the times indicated. Control cultures were incubated at 4° to permit measurement of passive surface adsorption of beads.

Following incubation, the medium was decanted and the cell sheet washed twice with Gey's balanced salt solution (GIBCO). Beads adsorbed to the cell surface were removed by incubation with a weak (0.05% w/v) solution of trypsin for 10 minutes at 37°. Following further washing with Gey's solution, the cells were detached with 0.25% (w/v) trypsin. An aliquot of the resulting suspension was removed for cell counting, while the remainder was centrifuged and the pellet resuspended in a solution of sodium dodecyl sulphate (1% w/v). The optical density at 500nm of the resulting suspension was recorded. Using a standard curve constructed by measuring the absorbance at 500nm of standard suspensions of cells and beads, it proved possible to calculate the average number of beads per cell.

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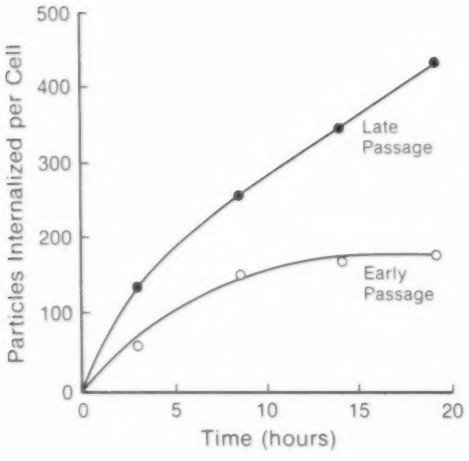

FIG. 1. Internalisation of latex beads by sub-confluent, monolayer cultures of early and late passage lapine articular chondrocytes.

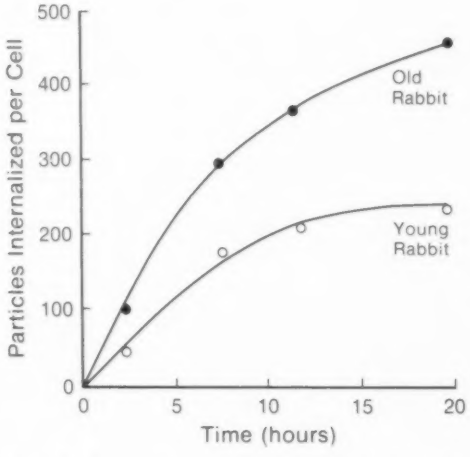

FIG. 2. Internalisation of latex beads by sub-confluent, monolayer cultures of first passage chondrocytes isolated from the articular cartilage of young and old rabbits.

RESULTS

Lapine chondrocytes of early passage cultures internalized less beads than those of late passage (Figure 1). Not only were the total number of intracellular particles lower in early passage cells, but the initial rate of uptake was lower. In addition, early passage cells appeared to saturate with beads earlier, and after a lower number of beads had been engulfed. These differences were not restricted to cells which had been reproductively aged *in vitro*. Primary monolayer cultures of articular chondrocytes from retired breeder rabbits, aged 3 years, showed a similar elevation in the rate and degree of endocytosis (Figure 2). In both cases, the rate of endocytosis was approximately doubled in old cells.

Chondrocytes isolated from old rats also internalized more beads than those cultured from young rats, although the enhancement was only 33% in this case (Table 1).

Early and late passage human fibroblasts were also tested for endocytic activity. In this case, the internalization of beads was assessed visually. Comparison of early and late passage cells in this respect left no doubt that the old fibroblasts were more phagocytically active (Figure 3). As a result of phagocytosis, the late passage cells became highly vacuolated.

TABLE 1. PHAGOCYTOSIS OF LATEX BEADS BY PRIMARY CULTURES OF ARTICULAR CHONDROCYTES FROM OLD AND YOUNG RATS.

		Beads per Cell			
		Young Cells		*Old Cells*	
4°	Flask 1	112		350	
			Average		Average
	Flask 2	83	100.3	307	337.7
	Flask 3	106		356	
37°	Flask 1	224		501	
			Average		Average
	Flask 2	221	223.3	471	501.3
	Flask 3	225		532	
Average beads internalised per cell:			123 (100%)		163.6 (133%)

Triplicate cultures of sub-confluent cells were incubated for 14 hours with latex beads at 4° or 37°.

DISCUSSION

Our data confirm the inference drawn from ultrastructural studies of *ex vivo* tissues, that chondrocytes have phagocytic ability. Endocytosis of latex beads by lapine chondrocytes approximately doubled during *in vitro* and *in vivo* aging. For articular chondrocytes of rats, the increase in endocytosis with age was a more modest 33%.

Repetition of the phenomenon with human fibroblasts eliminates several potential criticisms of the studies with chondrocytes. Chondrocytes have a tendency to dedifferentiate in culture, complicating interpretation of studies using cultured cells. This problem is not so acute with fibroblasts. Secondly, chondrocytes secrete an extracellular matrix which could act as a barrier to the phagocytosis of added beads. Declining matrix

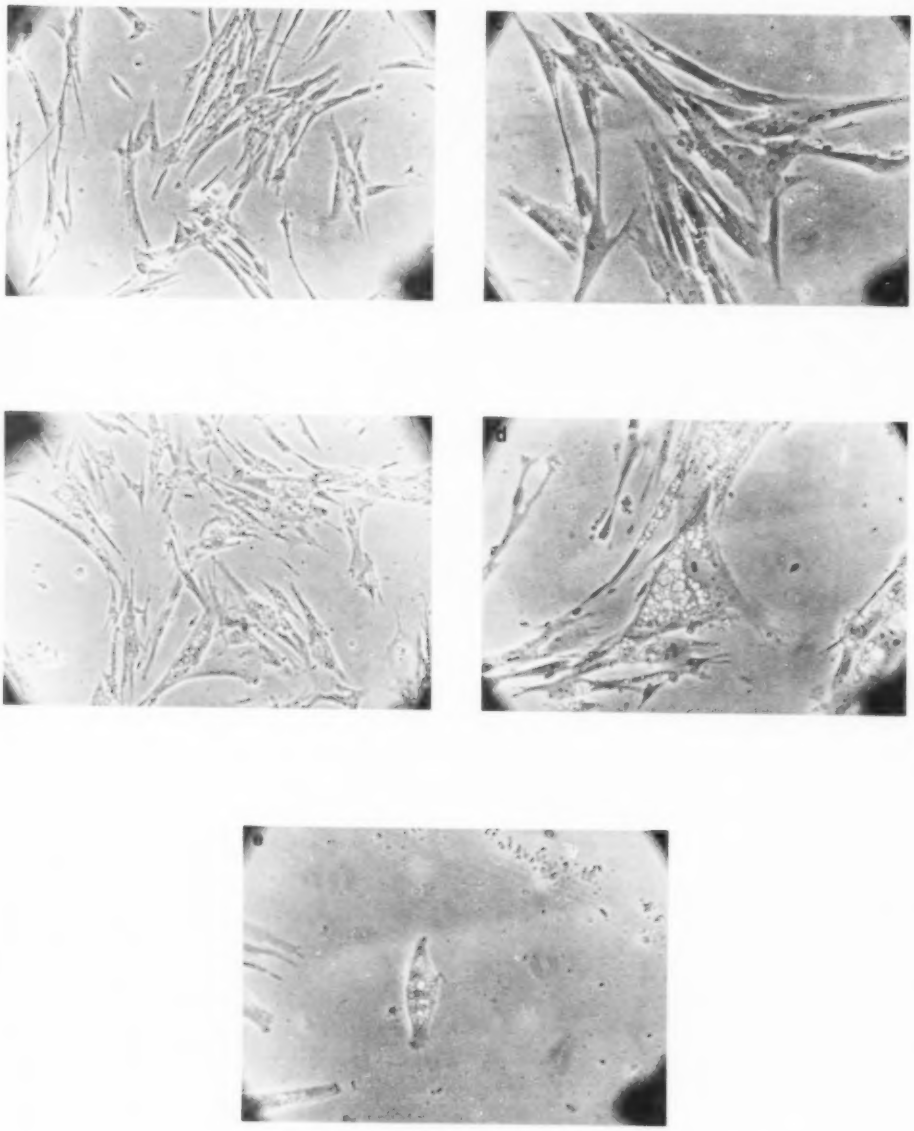

FIG. 3. Phase-contrast photomicrographs of early (PDL15) and late (PDL45) passage IMR90 cells following 18h incubation with latex beads. Surface-bound beads were removed by mild trypsinising and the cells replated, prior to photography.

a) Early passage cells; original magnification ×100.
b) Late passage cells; original magnification ×100.
c) Early passage cells; original magnification ×200.
d) Late passage cells; original magnification ×200.
e) Small, but phagocytic, late passage cell; original magnification ×100.

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production during aging *in vivo* (Madsen *et al.*, 1983) and *in vitro* (Evans and Georgescu, unpublished) could thus account for apparently greater phagocytosis of beads by senescing cells. That late passage fibroblasts also internalize more beads suggests that differential cartilaginous matrix production is not the cause. It does, however, remain likely that the increased size of the older cells makes an important contribution to the effect. This, however, may not provide a total explanation, as it was possible to identify small cells in late passage cultures which had nevertheless engulfed considerable numbers of beads (Figure 3e).

Of interest are reports that the phagocytic activities of macrophages derived from old mice (Watanabe and Russell, 1980) are enhanced. The phagocytic rates of cultured human blood monocytes (Gardner *et al.*, 1981) have been found to remain constant with age, however. A review of the recent literature on the subject (Brouwer and Knook, 1983) suggests that phagocytic rates of cells of the reticulendothelial system remain unchanged or increase with age. Previous reports on possible changes in the endocytic activities of other types of cell with age appear to be limited to that of Barnett *et al.* (1963), who noted the presence of collagenous filaments in the cytoplasm of old lapine chondrocytes. There are several reports that late passage human fibroblasts replicate viruses as efficiently as early passage, young cells (Holland *et al.*, 1973; Tomkins *et al.*, 1974; Pitha *et al.*, 1975). As endocytosis is a mechanism through which viruses penetrate cells, these studies suggest that this function is not impaired in late passage human fibroblasts.

The possibility that certain types of cell, under appropriate conditions, enhance their rates of endocytosis during aging is intriguing and suggests a possible common mechanism in diseases of old age in which there is an exaggerated breakdown of the extracellular compartment. That this is related to the increase in intracellular inclusions, the elevated lysosomal enzyme activity and enhanced vacuolation of old cells remains an attractive speculation. Phagocytosis of large numbers of latex beads promoted vacuolation in the late passage fibroblasts used here.

In addition to facilitating the direct breakdown of extracellular material by internalization and digestion in the lysosomes, phagocytic stimuli promote the secretion of neutral proteinases and other factors that accelerate the destruction of the extracellular matrix (e.g. Werb and Gordon, 1975; Werb and Reynolds, 1974; Evans *et al.*, 1981). In this manner, the effects of a small increment in endocytosis by an aging cell could become greatly magnified.

Articular cartilage lies vulnerable to small shifts in the metabolism of articular chondrocytes. An age-related increase in their catabolic activities would, by the mechanisms indicated above, promote degenerative changes in diarthrodial joints. Further research is necessary to establish whether this is so.

SUMMARY

We have compared the uptake of latex beads by cultures of old and young chondrocytes and fibroblasts. In each case, their phagocytic rates increased with cellular age.

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EFFECT OF STEROID HORMONES ON AGE DEPENDENT CHANGES IN RAT ARGINASE ISOENZYMES

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Abstract–Age dependent changes in rat liver and kidney arginase isoenzymes from day 1 to 73 weeks have been studied. It has been observed that total liver arginase activity (activity/liver) increases with age, but there is no appreciable change in the proportions of isoenzymes, their pH optima, electrophoretic mobilities and inhibition characteristics, although K_m increases with age. Corticosterone induces liver arginase activity and the degree of induction decreases with age. The hormone has no effect on kidney arginase. Testosterone treatment, on the other hand, induces kidney arginase without any effect on liver enzyme.

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LIGHTBODY (1938) WAS the first to study age-dependent changes in rat liver arginase, (E.C. 3.5.3.1), who observed an oscillating pattern of enzyme activity during development and a decrease in old age. Charbonneau *et al.* (1967) reported that the rat liver arginase activity was maximum when body weight reached about 100 g and then began to decrease. Highest activity both in liver and kidney cortex has been reported in six week-old rats (Shukla and Kanungo, 1969).

Isozymic forms of arginase was demonstrated by the use of DEAE cellulose columns (Gasiorowska *et al.* 1970). In liver they showed the presence of A_1 and A_3 forms and A_1 and A_4 in kidneys, A_1 being the main form of the enzyme in liver and A_4 in kidneys. The above observations were confirmed (Porembska *et al.*, 1971) using starch gel electrophoresis. All the above forms have similar mol. wts. (120,000) and almost similar affinities for the substrate. However, Rao and Kanungo (1974) have reported the same specific activity for liver arginase in 10 and 70 weeks old rats. They also found a single molecular form of the enzyme in these age groups.

The urea cycle enzymes are known to be inducible by nutrients like proteins, and hormones (Schimke, 1963; Ashida and Harper, 1961). The present studies were undertaken to see whether the age dependent changes in arginase could be related to the hormonal changes in the animals. Influence of two hormones, namely, corticosterone and testosterone has been studied.

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MATERIALS AND METHODS

The chemicals used were from the following sources: L-Arginine (BDH), DEAE-Cellulose (Bio Rad Laboratories, USA), corticosterone, testosterone propionate, acrylamide, biscrylamide and N.N.N'.N'-tetramethylene ethylene diamine (Sigma, USA). Other chemicals were of analytical grade made locally.

Experimental animals

The animals used for the study were male albino rats (Wistar strain). They were maintained on Hind Lever rat feed. Rats from day 1 to 73 weeks of age were used.

Tissues for enzyme study

Animals were killed by cervical dislocation, livers and kidneys were separated, washed thoroughly with cold 5 mM Tris-HCl buffer containing 5 mM $MnCl_2$ (pH 7.5) to remove blood, blotted and kept frozen till they were used.

Separation of arginase isoenzymes

The frozen tissues were weighed and homogenized with 5 volumes of solution containing 5 mM $MnCl_2$-100 mM KCl in 10 mM Tris-HCl buffer (pH 7.5) in a Teflon glass Potter type homogenizer for 1 minute. The homogenate is left for an hour at 4°C with gentle stirring and then kept at −10°C for 24 hours. It is then thawed, centrifuged at 4°C and the sediment extracted once more as described above. The combined extracts were dialysed overnight against Tris-HCl buffer (pH 8.3) containing 5 mM $MnCl_2$. The precipitate, if any, was centrifuged and the clear supernatant was subjected to DEAE-cellulose chromatography. The above procedure extracted almost complete arginase activity from tissues.

DEAE-cellulose column chromatography

This was done using a slightly modified procedure of Gasiorowska *et al.* (1970). The dialysed tissue extract was applied on to a DEAE-cellulose column (1 × 10 cms) previously equiliberated with 5 mM Tris-HCl buffer (pH 8.3). The elution was carried out with 50 ml of equiliberating buffer and 5 ml fractions were collected. Elution was continued with 50 ml of the buffer containing 0.15 M KCl in case of liver and with 0.25 M KCl in case of kidney. Each fraction was analysed for protein and arginase activity.

Arginase assay

Arginase activity was determined by incubating 0.1 ml of the respective tissue homogenate (0.5 percent in case of liver and 5 percent in case of kidney) with 250 μmoles of glycine-NaOH buffer pH 10.00 and 1 μmole of manganese chloride in a volume of 2.0 ml, for 15 minutes at 37°C, at the end of which 50 μmoles of arginine (0.5 ml pH 10.0) was added and incubation continued for 15 minutes more. The reaction was arrested by addition of 0.5 ml of 10% TCA and centrifuged at 3000 rpm for 10 minutes. Urea liberated was estimated, taking an appropriate aliquot of the supernatant, by diacetyl-monoxime method. One unit of arginase activity is equal to 1 μmole of urea formed.

Protein estimation

Proteins in tissue homogenates and column fractions were estimated according to Lowry *et al.* (1951).

Hormone treatment

Animals of various ages were injected (i.p.) with saline suspension of 3 mg/100 g body weight of corticosterone or 1 mg/100 g body weight of testosterone propionate for 7 days. The controls were treated appropriately.

Polyacrylamide gel electrophoresis

Gel electrophoresis was carried according to the procedure of Davis (1964) using 5% acrylamide gel at pH 8.3.

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RESULTS AND DISCUSSION

Age dependent changes

Table 1 shows that rat liver arginase activity increased slowly from day 1 to day 14, then a sudden increase was observed on day 21. From day 28 onwards, there were marginal changes in the enzyme activity, except at the eighth week. Though total liver arginase activity (units/liver) increased with age, when expressed per 100 g body weight, there was an increase up to eight weeks and a gradual decrease thereafter. The data closely agrees with earlier results (Greengard *et al.*, 1970; Porembska, 1973; and Lamers and Mooren, 1980).

Table 2 gives the kidney arginase activity at various ages. The activity/g tissue shows fluctuating pattern with age, but the total arginase activity (activity/pair of kidney) gradually increases and decreases at the 73rd week.

Figure 1 gives the pattern of chromatographic separation of liver arginase isoenzymes. The proportions of isoenzymes (Table 3) from day 1 to 73 weeks show marginal variations. This shows that both isoenzymes undergo simultaneous changes in their concentrations during the lifespan studied.

The pH profiles of liver isoenzymes, A_1 and A_3, isolated from rats of ages 7, 13, 25 and 73 weeks show similar pH optima which lies between 10.0–10.5, which is also the pH optima of the original homogenate.

The K_m values of A_1 and A_3 arginase isoenzymes (Table 4 and Figure 2) determined by

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TABLE 1. LIVER ARGINASE ACTIVITY AT VARIOUS AGES

Age	*Number of animals*	*Arginase activity (×10⁴) units/g/hr.*	*Arginase activity (×10⁴) units/liver/hr.*	*Arginase activity (×10⁴) units/hr./100 g body wt.*
1 day	10	0.81 ± 0.07	0.17 ± 0.06	2.98
7 days	6	0.70 ± 0.10	0.29 ± 0.04	2.10
14 days	6	0.91 ± 0.08	0.70 ± 0.04	2.70
21 days	5	3.63 ± 0.08	5.40 ± 0.26	14.20
28 days	5	3.56 ± 0.31	7.43 ± 0.45	15.20
7 weeks	6	3.40 ± 0.30	17.42 ± 1.10	18.30
8 weeks	5	5.55 ± 0.05	30.60 ± 3.00	24.20
13 weeks	5	3.40 ± 0.12	32.60 ± 1.60	19.20
25 weeks	5	4.10 ± 0.38	53.70 ± 3.70	16.90
73 weeks	3	3.54 ± 0.29	53.20 ± 2.40	14.20

TABLE 2. KIDNEY ARGINASE AT VARIOUS AGES

Age (weeks)	*Number of animals*	*Arginase activity (×10⁴) units/g/hr.*	*Total arginase activity (×10⁴) units/hr.*
7	6	0.13 ± 0.003	0.12
8	5	0.15 ± 0.005	0.17
13	5	0.11 ± 0.003	0.21
25	5	0.14 ± 0.009	0.29
73	3	0.11 ± 0.004	0.23

TABLE 3. DISTRIBUTION OF ARGINASE ISOENZYMES A_1 AND A_3 IN LIVER AT VARIOUS AGES

Age	A_1	A_3
1 day	80	20
7 days	80	20
14 days	90	10
21 days	85	15
28 days	85	15
7 weeks	90	10
8 weeks	90	10
13 weeks	80	20
25 weeks	80	20
73 weeks	80	20

The values are average of three experiments in each age group.

TABLE 4. K_m VALUES FOR A_1 AND A_3 LIVER ARGINASE ISOENZYMES AT VARIOUS AGES

Age (weeks)	A_1 *(mM)*	A_3 *(mM)*
7	6.6	7.7
25	12.5	12.5
73	20.00	16.6

Lineweaver-Burk plots show that the affinity of the substrate to the enzyme decreases with age.

The diamino acids, lysine and ornithine are competitive inhibitors of arginase (Gasioroskawa *et al.*, 1970). The inhibitory action of the above amino acids showed no age dependent changes.

Liver arginase isoenzymes A_1 and A_3 isolated from 7, 25 and 73 weeks rats on polyacrylamide disc electrophoresis at pH 8.3 showed that A_1 from all three ages studied remained near the origin and A_3 moved towards the anode. The mobility of A_1 and A_3 in all the ages studied showed that isoenzymes exist in the same molecular forms.

Figure 3 gives a pattern of chromatographic separation of kidney arginase isoenzymes A_1 and A_4. Like liver arginase, the kidney isoenzymes A_1 and A_4 do not show any appreciable age dependent variations (Table 5). The pH optima for A_1 are between 9.5–10.0, at all ages studied.

The above data clearly show that concentrations of arginase isoenzymes in liver and kidney remain almost constant and they may exist in the same molecular forms. As the synthesis of isoenzymes are under the control of different genes, from the above data, it is clear that the genes for A_1 and A_3 of liver and A_1 and A_4 of kidney remain unchanged throughout.

TABLE 5. DISTRIBUTION OF ARGINASE ISOENZYMES A_1 AND A_4 IN KIDNEYS AT VARIOUS AGES

Age (weeks)	A_1	A_4
7	9	91
13	5	95
25	5	95
73	5	95

The values are average of three experiments in each age group.

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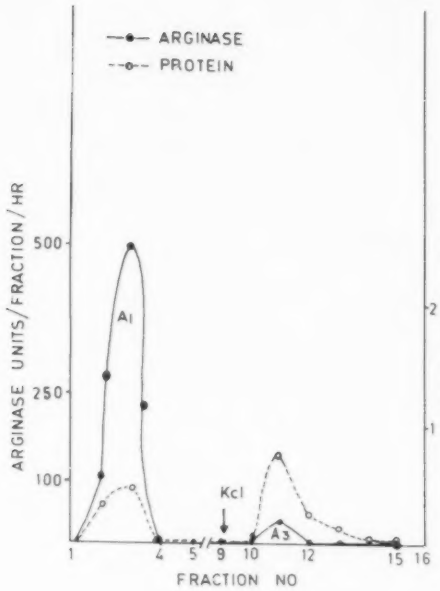

FIG. 1. Separation of Liver arginase isoenzymes on DEAE-Cellulose Column. Each fraction is 5 ml.

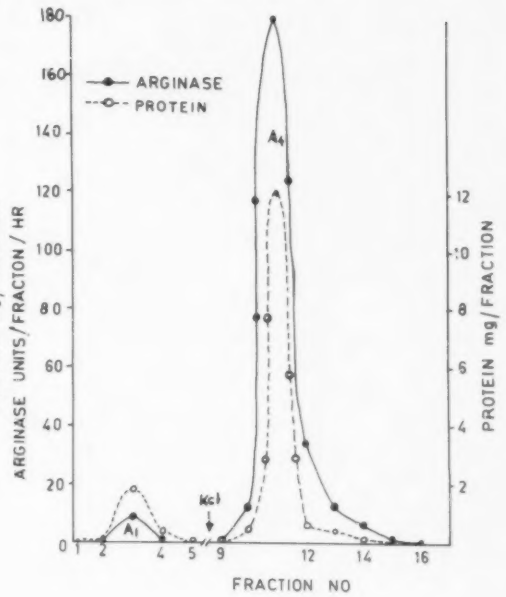

FIG. 3. Separation of Kidney arginase isoenzymes on DEAE-Cellulose Column. Each fraction is 5 ml.

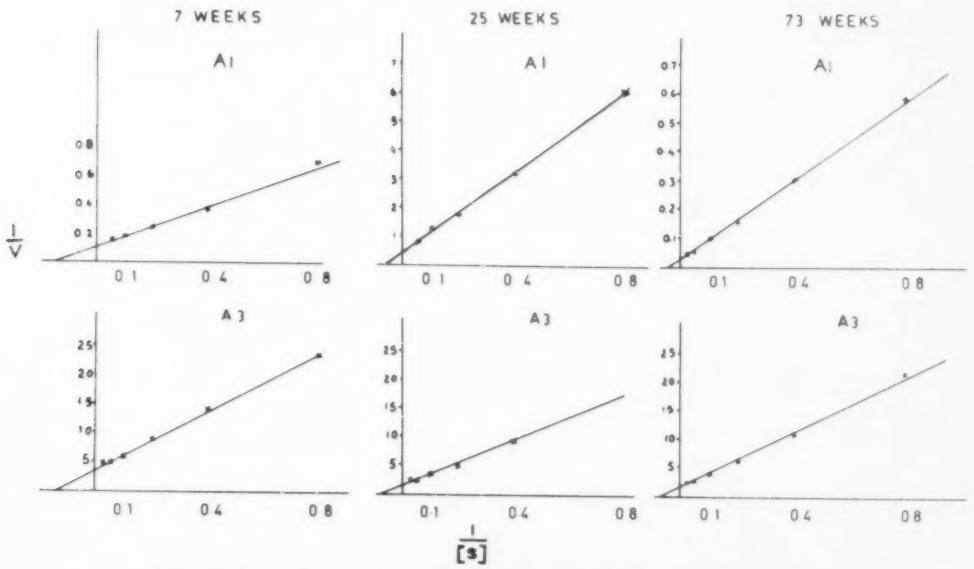

FIG. 2. Line Weaver-Burke Plots of A_1 and A_3 arginase isoenzymes from rat liver of various ages.

Effect of steroid hormones

Glucocorticoids elevate the urea cycle enzymes, secondary to protein catabolism. Greengard *et al.* (1970) studied the effect of hydrocortisone on the developmental formation of arginase in rat liver. They observed that after the age of 28 days a single dose of hydrocortisone did not cause any increase in the enzyme activity. They also concluded that induction of arginase in liver of adult rats requires high daily doses of hydrocortisone for a week. Berlin and Schimke (1965) observed that arginase responds very slowly either to the treatment with cortisone or to increase in dietary protein. They suggested that the enzymes which are in constant usage or whose physiological activity is controlled by availability of substrate would not be required to have the ability to fluctuate rapidly to any environmental stimulus. Rao and Kanungo (1974) did not observe any increase in the specific activity of liver arginase at 10 and 70 weeks after treating with cortisone (3 mg/100 g body weight) for three days. Probably this might be due to the limited time of hormone treatment. In view of the above variations, rats of ages 6, 12, 24 and 72 weeks were treated with corticosterone (3 mg/100 g body weight) for seven days.

Corticosterone treatment increases liver arginase activity (Table 6) in all the four age groups studied. But the degree of induction decreases from 98% at 7 weeks to 24% at 73 weeks, exhibiting an age dependent decrease in degree of induction. Our above data are in agreement with the observations of Rahman and Peraino (1973) on the induction of ornithine aminotransferase in response to high protein diet alone or in combination with glucocorticoids.

Isoenzymes from liver of corticosterone treated rat were separated on DEAE-cellulose column. The proportions of the isoenzymes do not (Table 7) show any change except in case of seven week rats where the proportions change from 90:10 to 80:20. The elution characteristics, pH optima and electrophoretic mobilities of these isoenzymes are similar to those of respective controls. The interesting observation in the present study is that

TABLE 6. EFFECT OF CORTICOSTERONE AND TESTOSTERONE ON LIVER AND KIDNEY ARGINASE AT VARIOUS AGES ($\times 10^4$ UNITS/G/TISSUE/HR.)

	Liver			*Kidney*		
Age (weeks)	*Normal*	*Corticosterone*[a]	*Testosterone*[b]	*Normal*	*Corticosterone*[a]	*Testosterone*[b]
7 (n = 5)	3.40 ± 0.30	6.73 ± 0.48 (+98%) P < 0.001	3.97 ± 0.08 P = NS	0.13 ± 0.003	0.14 ± 0.007 P = NS	0.25 ± 0.010 (+95%) P < 0.001
13 (n = 5)	3.40 ± 0.12	5.00 ± 0.14 (+47%) P < 0.001	3.41 ± 0.13 P = NS	0.11 ± 0.003	0.11 ± 0.006 P = NS	0.21 ± 0.008 (+94%) P < 0.001
25 (n = 5)	4.10 ± 0.38	5.60 ± 0.32 (+36%) P < 0.01	4.64 ± 0.44 P = NS	0.14 ± 0.009	0.15 ± 0.010 P = NS	0.27 ± 0.024 (+97%) P < 0.001
73 (n = 5)	3.54 ± 0.29	4.40 ± 0.19 (+24%) P < 0.05	3.65 ± 0.17 P = NS	0.11 ± 0.004	0.12 ± 0.005 P = NS	0.22 ± 0.010 (+100%) P < 0.001

[a]Corticosterone: 3 mg/100 g body weight for 7 days.
[b]Testosterone propionate: 1 mg/100 g body weight for 7 days.
NS = Not significant.

TABLE 7. VARIATIONS IN ARGINASE ISOENZYMES IN LIVER AFTER CORTICOSTERONE TREATMENT AND IN KIDNEY AFTER TESTOSTERONE TREATMENT

	Liver		*Kidney*	
Age (weeks)	*Normal* $A_1:A_3$	*Corticosterone* $A_1:A_3$	*Normal* $A_1:A_4$	*Testosterone* $A_1:A_4$
7	90:10	80:20	9:91	5:95
13	80:20	80:20	5:95	2:98
25	80:20	80:20	5:95	2:98
73	80:20	80:20	5:95	2:98

The values are average of three experiments in each age group.

though there is a decrease in degree of induction of enzyme activity with age, the proportions of isoenzymes do not show any change, indicating that both the isoenzymes are induced simultaneously with age.

Corticosterone at the given dose has no effect on kidney arginase.

In a variety of tissues, increase in arginase occur in association with growth. Such changes include the increase in kidney arginase with testosterone treatment, in liver of females with thyroxine, the increase in testes at puberty and the increase in liver and mammary gland during lactation. Kochakian (1945) and Frieden and Fischel (1968) have observed that administration of testosterone increases kidney arginase activity in rat and mice. Based on the above data, rats of 6, 12, 24 and 72 weeks were injected with 1 mg/100 g body weight of testosterone propionate to study the effect of hormone on kidney arginase with age.

Testosterone treatment doubles the kidney arginase activity (Table 6) in all the four ages studied, showing that age has no effect on the induction of enzyme activity in response to the hormone. Further, the proportions of kidney arginase isoenzymes (Table 7) show marginal changes in the major fraction. Thus, the kidney isoenzymes change simultaneously in all ages studied, as was observed with liver. The elution characteristics, pH optima of the above isoenzymes are similar to those from their respective controls.

Testosterone at the dose given has no effect on liver arginase activity in any of the four age groups studied.

Adelman (1975) classified the altered adaptive increase in the activities of a number of enzymes in response to a broad spectrum of stimuli into four categories. Some enzyme adaptations are altered in time course and/or magnitude of response whereas others for reasons not known are not altered at all.

Our data on the induction of liver arginase by corticosterone treatment resembles the second category of the above classification wherein the magnitude of response decreases with age. The data on the increase in kidney arginase in response to testosterone treatment is similar to the fourth category of the above classification, where the magnitude of response did not show any change with age.

SUMMARY

Age dependent changes in rat liver arginase activity from day 1 to 73 weeks show variations when the enzyme activity expressed per g of liver, but the total arginase activity (ac-

tivity/liver) increases with age. The proportions of arginase isoenzymes A_1 and A_3 in liver do not show any age dependent change. The pH optima, inhibition characteristics by diamino acids, the electrophoretic mobilities of the isoenzymes are same in all the ages studied, whereas K_m increases with age. Corticosterone induces liver arginase, but the degree of induction decreases with age from 98% at 7 weeks to 24% at 73 weeks. However, no change in the proportions of the isoenzymes or their physio-chemical characteristics is observed.

Kidney arginase activity from 7 weeks to 73 weeks shows variations with age, but the proportions of isoenzymes do not show any change. Testosterone induces kidney arginase to the same extent in all the ages studied. The proportions of kidney arginase isoenzymes A_1 and A_4 do not show any appreciable change with age after hormone treatment.

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CHANGES IN NUCLEIC ACID CONTENT IN AGING *DROSOPHILA BIPECTINATA*

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Abstract—DNA content decreases during first 20 and 25 days of survival in male and female flies of *Drosophila bipectinata* respectively. RNA as well as RNA/DNA content show a similar trend in males while in females both rise significantly during the first 5 to 10 days of survival followed by a steady decline till day 25. The study suggests that decreasing nucleic acid content of aging *D. bipectinata* is accompanied by the decline in protein synthesizing capacity.

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INTRODUCTION

AGING, LIKE growth and differentiation, is the outcome of sequential activation and repression of genes which leads to altered protein synthesis and thus the biological expression during different phases of life cycle (Kanungo, 1980). The studies on DNA and RNA are important to understand the molecular and genetic basis for a programmed development, maturation and senescence (Rockstein and Miquel, 1973).

Reports regarding the age-related variations in nucleic acids are contradictory. Balazs and Haranghy (1965) and Lang *et al.* (1965) reported increase in nucleic acid content during first five days of survival in *D. melanogaster* and *A. aegypti* respectively. Yale and Ballard (1966) noted the constant levels of nucleic acids throughout the lifespan in houseflies. Samis *et al.* (1971), King (1973), Massie *et al.* (1975, 1981), Wintzerith (1978), Kara and Patnaik (1982), and Sharma *et al.* (1984) have reported age-related decline in nucleic acid content in different systems in various animals. The present communication is to study age-related quantitative variations of nucleic acids and thus to evaluate the protein synthesizing capacity during different phases of the life cycle in *Drosophila bipectinata*.

MATERIAL AND METHODS

Rearing of insects

Stock cultures of *D. bipectinata* (Duda) were maintained constantly at 26 ± 2°C on standard medium composed of maize flour, agar agar, brown sugar, dried yeast (72:24:64:24) in culture bottles (250 ml capacity). Newly emerged flies were collected at 24 hour intervals and were maintained till 25 days of age. The flies were transferred to fresh food medium every tenth day to avoid the mixing of old culture with newly emerging flies. Biochemical assay was performed in male and female flies separately by sexing them on the basis of external morphological characters (Rockstein and Miquel, 1973). The mean lifespan of male and female flies at 26 ± 2°C is 21 and 23 days respectively (Sharma and Wadhwa, 1983).

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Chemicals

Trichloroacetic acid (TCA) ethanol, perchloroacetic acid (PCA), diphenylamine (DPA), glacial acetic acid (GA), sulphuric acid (H_2SO_4), orcinol, hydrochloric acid (HCl), Ferric chloride ($FeCl_3$), deoxyribose and ribose sugars were purchased from Glaxo BDH Chemicals and Sigma Chemicals.

Preparation of homogenates

Male and female flies of different age groups (1, 5, 10, 15, 20 and 25 days) were weighed and crushed separately in cold distilled water at 4°C to prepare 15% w/v homogenates.

Extraction and estimation of nucleic acids

The homogenate (1 ml) was treated with 10% cold TCA (1ml), centrifuged at 3500 rpm for 10 minutes and the supernatant was discarded. The lipids were removed by treating the residue with 95% ethanol (1 ml). Nucleic acids were extracted by suspending the residue to 0.5 N PCA (1.3 ml). The mixture was heated for 1 hour and 30 minutes successively at 80°C in a water bath. The supernatant obtained in two successive treatments contained DNA and RNA which were estimated by the colorimetric reactions with DPA and orcinol respectively as suggested by Schneider (1957). The standard curves of DNA and RNA were prepared by taking different concentrations of deoxyribose and ribose sugars. The significance of the results was tested by applying student's *t*-test.

RESULTS

The DNA content (μg DNA/100 mg tissue ± S.E.) of males is lower than that of females of comparable age groups (Table 1). It declines with age in both the sexes (30.05%) (147.07 ± 2.69 to 102.20 ± 1.08) during first 20 days of male survival and (43.95%) (195.80 ± 2.14 to 109.73 ± 2.89) during first 25 days of female survival. The decline is rapid during first 5 days (14.41%) followed by 2.56%, 6.31% and 11.04% during successive age intervals each of 5 days till 20 days in males (Table 1). Similarly in females the percentage decline was 16.92 during first 5 days followed by 16.67, 8.32, 7.14 and 4.48 during successive age intervals each of 5 days till 25 days (Table 1).

TABLE 1. DEOXYRIBONUCLEIC ACID CONTENT IN AGING *D. BIPECTINATA*

	Males		*Females*	
Age in days	*μg DNA/100 mg tissue ± S.E.*	*Per cent decrease*	*μg/DNA/100 mg tissue ± S.E.*	*Per cent decrease*
1	147.07 ± 2.69		195.80 ± 2.14	
	$p < .001$	14.41	$p < .001$	16.92
5	125.87 ± 1.44		162.67 ± 2.90	
	NS	2.56	$p < .05$	16.67
10	122.64 ± 2.36		135.55 ± 2.97	
	$p < .05$	6.31	$p < .05$	8.32
15	114.90 ± 1.18		124.26 ± 2.74	
	$p < .001$	11.04	$p < .05$	7.14
20	102.20 ± 1.08		115.38 ± 1.69	
			NS	4.48
25			109.73 ± 2.89	

n = 6
$p < .001$ very significant
$p < .05$ significant
NS Not significant at 0.05 confidence level

TABLE 2. RIBONUCLEIC ACID CONTENT IN AGING *D. BIPECTINATA*

Age in days	*Males*		*Females*	
	μg RNA/100 mg tissue	*Per cent decrease*	*μg RNA/100 mg tissue*	*Per cent decrease/increase*
1	215.92 ± 1.26		217.66 ± 1.61	
	$p < .001$	40.37	$p < .001$	+18.46
5	128.73 ± 0.89		257.86 ± 1.81	
	$p < .001$	10.00	$p < .001$	−7.52
10	107.21 ± 1.86		238.45 ± 2.42	
	$p < .05$	2.33	$p < .001$	−22.52
15	102.13 ± 0.87		184.73 ± 1.82	
	$p < .001$	5.13	$p < .001$	−34.70
20	90.11 ± 0.96		120.61 ± 2.72	
			$p < .05$	−6.32
25			112.99 ± 1.01	

n = 6
$p < .001$ very significant
$p < .05$ significant
NS Not significant at 0.05 confidence level

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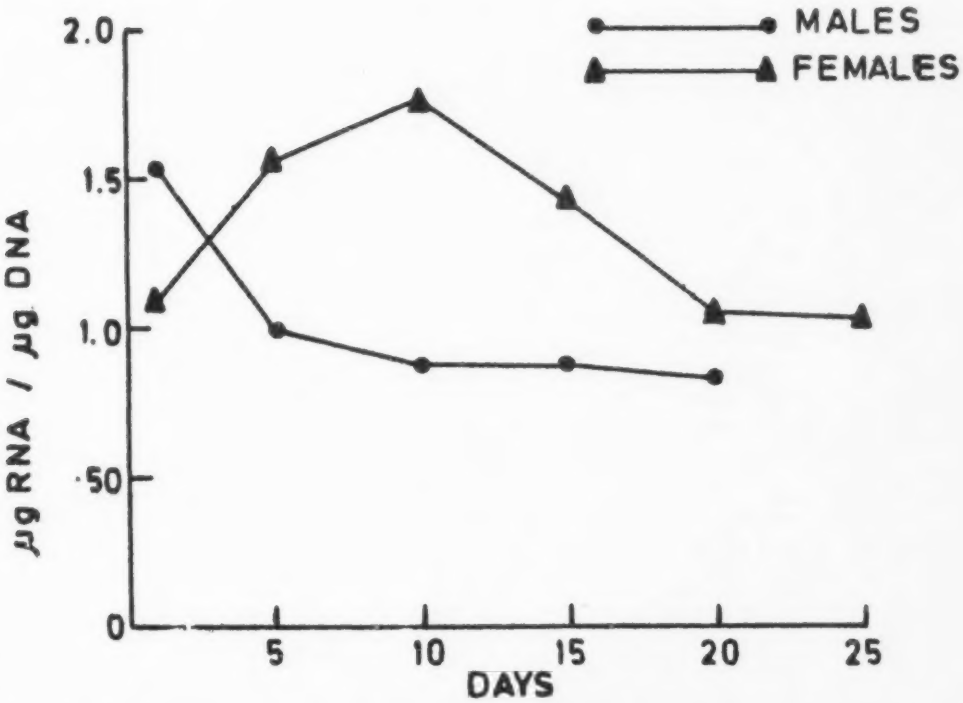

FIG. 1. Protein synthesizing capacity (μgRNA/μgDNA) in aging *D. bipectinata*.

RNA content (μg RNA/100 mg tissue ± S.E.) was also lower in males at all age intervals when compared with females (Table 2). It decreases continuously with age in males (40.37%, 50.37%, 52.70% and 57.83% during first 5, 10, 15 and 20 days of survival respectively). In females the RNA content shows a leap of 18.46% (217 ± 1.61 to 257.86 ± 1.81) during first 5 days of survival (Table 2). During next 5 days it decreases by 7.52% and the increase during first 10 days of survival is left to be 9.55%. Thereafter occurs a steady decline (22.52%, 34.70%, 6.32% in successive age intervals each of 5 days till 25 days (Table 2).

RNA/DNA ratio declines from 1.542 ± 0.024 to 0.8824 ± 0.003 (42.71%, $p < .001$) in first 10 days in males (Figure 1). Total decline during 20 days of survival in males is 44.46% (1.5422 ± 0.024 to 0.8564 ± 0.020, p .001). In females the ratio increases by 41.70% and 58.13% (1.1082 ± 0.007 to 1.5704 < 0.029 to 1.7524 ± 0.020) in first 5 and 10 days of survival respectively. Afterwards, the content decreases by 17.54% $p < .001$, 26.11% $p < .001$ and 2.05 p − NS in successive age intervals each of 5 days till 25 days (Figure 1). The total decline in females was only 5.61% during 25 days of survival ($p < .001$).

DISCUSSION

Lang *et al.* (1965) and Blevins (1973) reported that DNA content is the index of the estimate of cell number. But the DNA content measured as per wet weight basis reflects the total DNA which includes the nuclear DNA and the mitochondrial DNA as well. So the amount of DNA in the present case in *D. bipectinata* is not directly related to cell number. DNA content of males is lower than that of females at all age groups. This difference is attributable to the difference in their mean wet weight as also suggested by Samis *et al.* (1971) in *D. melanogaster.* The total DNA decline with age can be due to many factors, namely, loss of cell number (Blevins, 1973), loss of mitochondria (Massie *et al.*, 1981; Takahashi *et al.*, 1970b; Sacktor and Shimada, 1972), decline in the efficiency of normal DNA repair process thus adding the random lesions in nuclear as well as mitochondrial DNA leading to their degradation and loss (Little, 1976).

RNA content is a measure of potential rate of protein synthesis and RNA/DNA ratio is an index of protein synthesizing capacity/cell (Lang *et al.*, 1965 and Ring, 1973). Both the parameters are seen to increase significantly during first 5 to 10 days (reproductive period) in females of *D. bipectinata.* The increased amount of proteins are possibly required for reproductive demands which are agreed to be much more in females as compared to males (Samis *et al.*, 1971; Sharma and Sharma, 1979; Sharma *et al.*, 1984). Lower RNA content of males as compared to females at all age intervals suggests that females are more efficient in synthesizing and retaining proteins throughout their lifespan. This can be supported by the results of Chen and Levenbook (1966) and Blevins (1973). Results of RNA/DNA content suggests that the males are fully mature at one day age while females become mature on tenth day only when they attain a peak of protein synthesizing capacity. Similar report has been given by Sharma *et al.* (1984) in *Callosobruchus maculatus.*

In conclusion the aging in *D. bipectinata* is accompanied by significant decline in nucleic acid content and protein synthesizing capacity.

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DOES A RELATIONSHIP EXIST BETWEEN SPONTANEOUS LOCOMOTOR ACTIVITY, FITNESS AND LIFESPAN IN *DROSOPHILA MELANOGASTER?*

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Abstract—Spontaneous locomotor activity (SLA), fecundity, fertility and lifespan were measured in a wild laboratory strain of *Drosophila melanogaster* in order to ascertain whether there is a genetic correlation between these traits. In females there is no correlation either between SLA and fecundity or between SLA and lifespan. It is concluded that, although a high level of SLA probably constitutes a favorable behavioral component of fitness, this does not mean that more active females have a larger number of offspring and live longer than less active ones.

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INTRODUCTION

In 1959 Manning studied the sexual behavior of *Drosophila melanogaster* and *Drosophila simulans*. He noted that while the sexual behavior of both species could be readily considered as being basically identical, *Drosophila melanogaster* was however more active and its courtship more vigorous than that of *Drosophila simulans*. He concluded that the changes in courtship behavior which he observed probably resulted from selection acting upon a common ancestral population so as to favor high activity in some environments and low activity in others.

Ewing (1963) accepted that opinion and further argued that it was not difficult to imagine ways in which changes in activity level could be of adaptive significance to a species. He supposed that the level of activity was to some extent under genetic control and tried to demonstrate it by selecting for spontaneous locomotor activity (SLA) in *Drosophila melanogaster*. He could however not reach that goal for he selected for reactivity instead of for activity.

Connolly (1966a) provided some interesting evidence in favor of the idea that a high level of locomotor activity is of adaptive significance. Indeed he could demonstrate that *Drosophila melanogaster* imagoes deprived of food are substantially more active than normally fed flies. (Both types of flies were observed for locomotor activity a short time

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after having been submitted to a mechanical stimulation.) It seems indeed obvious that the best way to find food is not to stay motionless but to be on the move.

Angus (1974a) tried to determine whether the measures of behavior of individual *Drosophila* are related to the behavior of a group in a relatively large space, a biologically meaningful situation. This comparison was made by taking two lines which had been clearly distinguished by two different techniques for measuring individual locomotor activity, and determining whether there was a similar distinction between them when measured in a group. His data show that the distinction between lines is only apparent when a small quantity of food is available. This confirms the findings of Connolly (1966a) and furthermore suggests that a high level of locomotor activity does indeed constitute a behavioral component of fitness.

Such indirect evidence does however not constitute a demonstration that the level of SLA is of adaptive significance. One should know to which form of genetic control that trait is eventually submitted. Médioni (1977) has convincingly argued that when a trait contributes to the overall fitness the additive components of the genetic variance are of a lesser importance than the non-additive ones.

Connolly (1966b) was able to select for high and low activity in *Drosophila melanogaster*. Although the methodology which he used has been criticized by Angus (1974a)–for the reason that Connolly has apparently been selecting for both reactivity and activity–it seems however likely that Connolly was able to select for activity (Connolly, 1967). If so it demonstrates that some additive components of the genetic variance are controlling SLA. It must however be stressed that, using a different methodological approach, Lints *et al.* (1984a) were unable to confirm Connolly's findings.

Hay (1972) claimed to have found evidence for directional dominance for high activity in *Drosophila melanogaster*. Yet his data, because of a bias generated by the experimental procedure used, are not reliable, as correctly observed by Angus (1974a). For his part Angus (1974b) analyzing the genetic control of SLA in *Drosophila melanogaster* claimed that a model including epistasis, as well as additive and dominance components fitted best to his data. He further deemed that such a genetic architecture for SLA was an indication of a history of natural selection for a comparatively high activity. To test that hypothesis Angus (1974c) compared during a year, at regular three-months intervals, the level of SLA of a F_2 generation issued from a cross between two inbred lines of *Drosophila melanogaster* to that of a population initiated from an identical F_2 generation and maintained by random mating in a population cage. (The F_2 generation may be regarded as having the same genetical constitution as the base population maintained in the population cage; it may therefore be used as a control with which the cage population may be compared.) Angus observed that the population maintained in the cage changed with the passing time towards an increased level of activity. As that population had been maintained in a constant environment Angus concluded that it was reasonable to suppose that this change was due to natural selection.

It thus appears likely that a high level of SLA constitutes a behavioral component of fitness. The question of the precise genetic control of that trait remains however open. Unanswered remains also the question of an eventual relationship between SLA and other traits related to fitness, such as fecundity, fertility and–although the relation between that last trait and fitness could be disputed–lifespan. The purpose of this paper is to look for such an eventual link. In other words to answer a simple question: do flies with a high level of SLA have more offspring and live longer than less active ones?

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MATERIAL AND METHODS

The *Drosophila melanogaster* strain used in the present experiment (UBAG) was derived from four *Drosophila* wild-type laboratory strains (Urbana, Bierbeek, Abeele, Gabarros) of various descents, maintained by mass-mating for several years in the laboratory at a constant temperature of 25 ± 0.5°C (for further details, see Le Bourg and Lints, in press).

The experimental flies were obtained in the following way. Flies of the UBAG strain, aged 5 to 6 days, were allowed to lay eggs during a 24 hr period. Eggs were then transferred in batches of 20 to vials containing about 15 cm^3 of the ordinary culture medium and live yeast in abundance. At emergence, virgin males and females were collected and transferred in groups of 50 flies of the same sex to half-pint bottles containing the culture medium. These flies constitute the experimental group of animals. The temperature of the breeding room was 25 ± 0.5°C. The room was lit for 12 hours a day, from 8 a.m. to 8 p.m.

The procedure to measure SLA has been described previously (Le Bourg and Lints, in press). Briefly, 24 hours before observation, flies are individually transferred to Petri dishes containing the usual culture medium. The behavior of the flies is observed from 8 a.m. to 8 p.m., approximately. Each fly is observed in turn, during a 5 seconds period, and recorded as active or inactive. Activity is quoted 1, inactivity 0. With that method it is possible to obtain 75 observations for each fly, 100 flies (50 flies, 9 days old, for each sex) being observed during the photophase of 12 hours. The individual scores of activity may thus vary between 0 (no observed activity) and 75 (fly active during each period of observation).

Thereafter the flies were crossed according to their rank of activity, the most active male being crossed with the most active female, and so on. Fecundity (number of eggs laid per female) was measured during three days starting with the 11th day of age. The eggs laid by each female were transferred in batches of 20 to vials containing 15 cm^3 of the usual culture medium and live yeast in abundance. At emergence, the imagoes were counted and their sex determined; that procedure allows fertility to be determined. In order to determine lifespan, the experimental flies already used were kept until death occurred; their vials were renewed thrice a week; the dead flies were recorded every day.

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RESULTS AND DISCUSSION

The estimation of the rhythm of SLA during the photophase is worked out in the following way. For each round of observations the percentage of active flies is computed; this computation is made for the 75 rounds for which we accumulated data. The rhythm of SLA is reported in Figure 1 (A for males; B for females). On the whole the pattern of

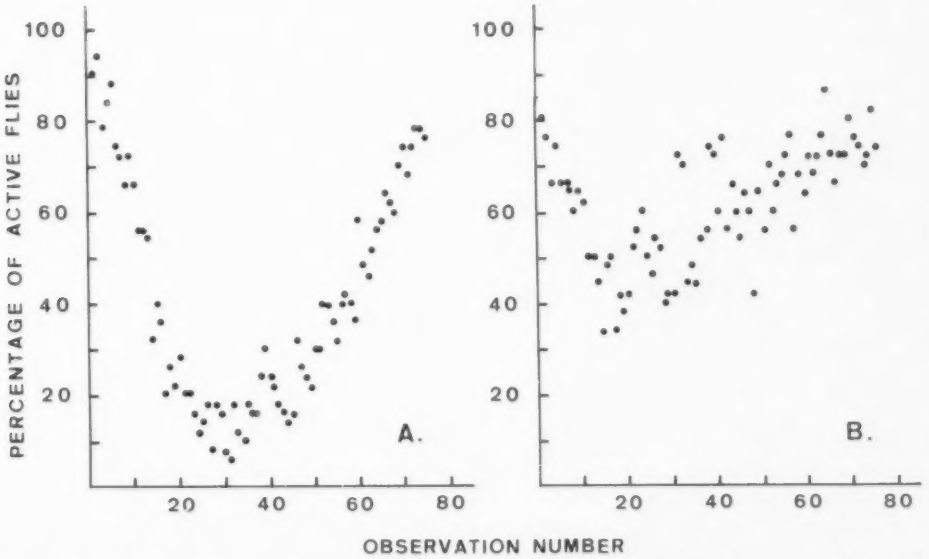

FIG. 1. Rhythm of spontaneous locomotor activity in males and females of a wild strain of *Drosophila melanogaster* during a 12 hr photophase. (A) males, n = 50, age = 9 days. (B) females, n = 50, age = 9 days.

the rhythm of SLA, observed in the present experiment, is similar to that previously described (Le Bourg and Lints, in press).

The survival curves for both males and females are reported in Figure 2. For males 41 flies are taken into account; 9 male flies have indeed been lost during the different manipulations. For females, n = 43, 7 flies having been lost in the course of the experiment. The mean lifespan (±S.E.) of females equals 46.9 ± 2.3 days and that of males 49.9 ± 3.3 days. The difference between the two sexes is not significant (Mann-Whitney test, Z = 1.03, N.S.). As emphasized by Lints *et al.* (1983) in *Drosophila melanogaster* the greater lifespan of females is not a rule.

The Spearman rank correlation coefficients between the various traits measured in females are reported in Table 1.

The correlation between the scores of SLA and fecundity (number of eggs laid during three days) is not significant. The same is true for the correlation between the scores of SLA and fertility (ratio of the number of live imagoes and the number of laid eggs). There is thus no direct relation between SLA, a probable behavioral component of fitness, and fecundity and fertility, two major components of fitness. That conclusion could eventually be disputed for, in the present experiment, fecundity and fertility were measured during only three days. However it seems reasonable to argue that if there was a correlation between SLA and fecundity and fertility, that correlation would have been detected with the experimental protocol used. It may indeed be assumed that, in the field, mainly because of predators, the length of the period of egg-laying is rather short; in other words, that natural selection probably favored an early expression of fitness (Williams, 1957). Therefore, if there was a link between SLA and fecundity or fertility or, in other words, if more active females were yielding more offspring than less active ones, that link should be

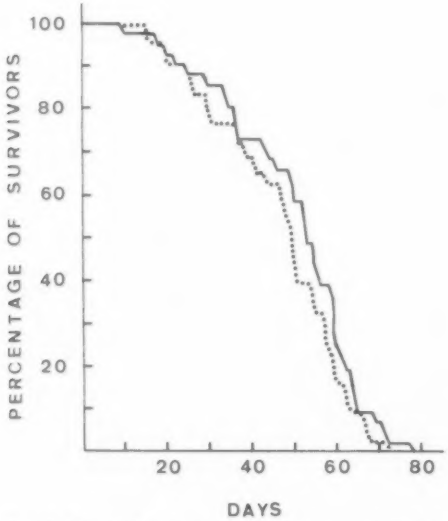

FIG. 2. Survival curves of the males (——) and the females (– – – –) observed for spontaneous locomotor activity. n = 43 and 41 for females and males, respectively.

TABLE 1. SPEARMAN RANK CORRELATION COEFFICIENTS BETWEEN THE SCORES OF SPONTANEOUS LOCOMOTOR ACTIVITY (SLA), FECUNDITY (FEC), FERTILITY (FER) AND LIFE-SPAN (LIF). N = 43 FEMALES.

	SLA	*FEC*	*FER*	*LIF*
SLA	1	0.133 (NS)	−0.008 (NS)	0.105 (NS)
FEC		1	0.295 ($p < 5\%$)	0.047 (NS)
FER			1	0.122 (NS)
LIF				1

apparent at young age; a high score of SLA should be concomitant with a high fecundity and a high fertility. Such a correlation does not exist.

The correlation between fecundity and fertility is positive and significant; in other words, females which lay the most also lay the most viable eggs.

For females, the correlation between SLA and lifespan is not significant (Table 1). The same is true for males (Spearman rank correlation coefficient, $n = 41$, $Z = -0.003$, N.S.). It has been suggested by Sohal (1976) that less active flies would eventually survive for a longer time. In *Musca domestica* several studies have been performed to test that hypothesis. They showed that when physical activity is increased–for instance, by increasing crowding, by harassing females by a large number of males, or *vice-versa*–lifespan decreases (Ragland and Sohal, 1973, 1975). In these experiments the level of physical activity has actually not been measured, but it has been deemed, in all likelihood, that it was higher in some environmental conditions. If it is true then these findings probably relate to a simple phenomenon of exhaustion. Yet when a correlation is sought at the individual level between measured SLA and lifespan (Sohal and Buchan, 1981) it is found to be non-significant (Lints, 1983), although the authors suggested that "the levels of physical activity of individual flies did, in general, correspond to the lifespan of the flies." Recently in a large study made on 600 individuals of *Drosophila melanogaster,* of both sexes and of three widely different genotypes (Lints *et al.,* 1984b) it was shown that there is, either at the populational or at the individual level, no relation between SLA and duration of life. The present findings confirm that result.

Finally the correlations between lifespan and fertility, on the one hand, and lifespan and fecundity, on the other hand, are non-significant. Giesel (1979), comparing 7 strains of *Drosophila melanogaster,* each strain being genetically homogeneous–4 isogenic lines and 3 F_1 hybrid lines–has shown that, *among* strains, there is a positive and significant correlation between lifespan and various expressions of fecundity and fertility. However the *within* strain correlations, that is the environmental ones, (since all flies within a strain are of the same genotype) between age of death and the various fecundity indices were non-significant. That last result confirmed data obtained by Lints and Lints (1971) with a genetically homogeneous heterozygous strain. In the present experiment the within strain non-significant correlation relates to a genetically heterogeneous strain; it is thus not purely environmental. The absence of a significant correlation is at variance with the results of Giesel. It could however be objected that a measurement of fecundity and fertility made at an early age but during three days only is not sufficient to obtain a good estimation of fecundity. This is true, except if the pleiotropy theory for the evolution of senescence is correct (Williams, 1957). Indeed Williams suggested that senescence could have evolved

by selection of genes that have different effects on fitness at different ages and more precisely of pleiotropic genes with beneficial effects in the first stages of life history and with deleterious effects in later life. On the basis of Williams' theory a negative correlation could be expected between early fecundity and lifespan. In all fairness it must however be noted that no definite proof for the exactness of Williams' theory has yet been given (cf. Rose and Charlesworth, 1980, 1981 and Lints, 1983).

Our results show that there is apparently no genetic correlation either between the level of spontaneous locomotor activity and lifespan, or between that level and two paramount components of fitness, namely fecundity and fertility. This does, of course, not mean that spontaneous locomotor activity does not constitute a behavioral component of fitness, although, as shown in the introduction, the evidence in favor of that idea remains scarce and, to some extent, contradictory.

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CHRONOLOGICAL AND BIOLOGICAL AGE

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Abstract—Resuming the thread of previous studies, this paper reports a new method of assessing biological age with the inclusion of physical, psychical, and social parameters. The suitability of the method was tested on a total of three hundred and sixty-five subjects. The results obtained allowed a reference curve to be plotted which can be considered a basis for an assessment of biological age.

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INTRODUCTION

AT A congress held in Kiev in 1963 the European Bureau of the World Health Organization called upon gerontologists of all disciplines to find suitable methods of determining the biological age in man. From a review of the gerontological literature it is, however, evident that this appeal was followed only by a small number of study groups. Although the subject was repeatedly discussed at national and international conferences such as the 1972 and 1981 world congresses held in Kiev and Hamburg, respectively, the number of those attending the respective symposiums was limited, leading to the conclusion that interest in the subject also is limited, at least in comparison with that shown in other subjects.

In 1972 our working group started reporting efforts directed toward assessing biological age in man (Ries, 1972). Studies were continued consistently, and a comprehensive report of the results obtained was published in 1982 (Ries, 1982). Meanwhile, new results have been obtained on the basis of further theoretical considerations and widened spectra of methods, which are here set out for discussion.

TERMINOLOGY

A description of terminological problems has been published only recently (Ries, 1983). However, it appears necessary for a few additions to be made to the definition of the chronological and biological age.

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Chronological age

The chronological or calendar age is a record of time elapsed since birth. This formulation is in accord with Bürger's (1960) classic definition of aging as a function of time. The chronological age which is determined by time is characterized by, and replete with, biological changes that range from conception to death in the sense of Bürger's (1956) biomorphosis. However, to exactly define these changes at a certain point in time is tantamount to an assessment of biological age.

Biological age

A uniform or generally accepted definition of the term "biological age" cannot be found in the gerontological literature. From a review by Čebotarev and Minc (1978) it is clear that it is possible for two different approaches to be adopted in defining the said term.

1. Biological age is considered an expression of typical phenomena of a normal course of life, that is, of age *per se*. According to this concept, only those characteristics should be used for its assessment which are known to undergo changes, in accordance with certain laws, in the course of life and which, in so far as their dynamics are concerned, lie outside of the spreads of age groups. All random characteristics including pathological phenomena cannot therefore be included in a spectrum of methods. According to these reflections, observance of such rules leads to a quantity that may be referred to as "orthological" age which is defined as follows: *Orthological age is a term used to describe the general condition of an individual at a certain time of his chronological age, which is marked by "normal" characteristics.*

2. The determination of the orthological age doubtless is of considerable importance to the description of alterations with aging, but does not, in our opinion, meet the requirements which, from a clinical point of view, must be generally made of an assessment of the biological condition of an organism. It is, therefore, considered that both orthological and pathological findings should be considered in any attempt at assessing biological age. Accordingly, we offer the following definition of what is known as biological age: *Biological age is a term used to describe the general condition of an individual at a certain time of his chronological age, which is marked by orthological and pathological characteristics.* Adoption of such an approach may, of course, result in a subject whose calendar age is twenty years showing the biological age of a fifty-year-old subject.

Also of major importance in this context is the question as to which categories of life have to be included in biological age. In the literature, a distinction is sometimes made between biological age and psychical age. By contrast, we have always held the view that an assessment of biological age requires that the entire human organism, including psychological parameters, be considered. In this sense, therefore, it is considered to be justifiable for biological age to be defined as the general condition of an individual at a certain time of his chronological age, which is characterized by physical and psychical features. This amounts to throwing light upon, and arresting, a certain moment in a human being's life, similar to a still taken from a motion picture.

However, it is necessary for this particular definition of biological age to be expanded in order to prevent it from being stuck in biological modes of thought. Going beyond Bürger's conceptions, aging today is considered to be a function not only of time but of space as well. Consequently, it is necessary to also use socially oriented parameters for an assessment of biological age. As with psychometric methods, the principal difficulty is establishing the quantifiability of suitable parameters.

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Summing up, biological age is, in this particular sense, considered a complex of physical, psychical, and social data. The corresponding definition is worded as follows: *Biological age characterizes the general condition of an individual at a certain time of his chronological age, which is marked by physical, psychical, and social characteristics.* It should be recalled that the WHO definition of the term health, by including physical, psychical, and social wellbeing, contains exactly the same categories.

METHODOLOGY

From what has been said above it seems clear that biological age must be regarded as a quantity that can be determined only through the use of a program of complex examinations.

A comprehensive test battery comprising a total of sixty parameters has been recommended by Comfort in 1969. This battery contains not only elements of Hollingsworth's (1965) Hiroshima study but also fundamental information supplied by Gitman (1969) who had devised a clinical screening method. The proposal made by Comfort (1969) also included psychometric tests and biopsic methods, but has, to our knowledge, never been used on a larger scale. The Rumanians Ciucă and Jucovski (1969) also included socioeconomic factors in their studies. Detailed information on previous efforts to assess biological age in different disciplines and branches of science has been reported elsewhere (Ries, 1982).

In our own studies, efforts have been made to arrive, in various series of experiments, at a more and more perfect synthesis of suitable parameters. The method devised by Pöthig comprises the following characteristics from three areas, it being of course entirely possible for partial overlapping to occur.

Physical area

Blood pressure (systolic, diastolic)
Vital capacity
Arterial oxygen partial pressure
Pulse rate during exercise
Vision
Hearing acuity
Hand grip strength
Tendon extensibility
Decayed, missing, and filled teeth

Psychical area

Color word test
Landolt's concentration time test
Optical response time
Acoustical response time
Viseomotor coordination ability
Basic rate of psychomotor activity
Labyrinth learning test
Subjective state of health
Self-control
Extroversion, introversion, rigidity

Social area

Social resonance
Social dominance
Social potency
Social activity

It is now clear from the results of extensive preliminary studies that this battery of tests is readily realizable, the time of testing being between sixty and one hundred and twenty minutes. References to the methods used are made elsewhere (Ries, 1982).

CALCULATION

What proved quite difficult was the mathematical evaluation and compilation of the large number of individual findings, especially as suitable models were not available. For example, the often-quoted paper by Bourlière (1970) contains only an enumeration of individual findings, without any attempt being made to assemble separate or subordinate parts into a whole.

The experience gathered thus far warrants the following approach to be recommended:

1. For a normalization of the individual parameters it is necessary to fix, for each method, a most favorable value (**G**) and a most unfavorable value (**U**). Using these quantities, the value (**W**) measured in an individual case can be converted to a partial index (**TI**) that enables the individual parameters to be compared.

$$\text{Formula:} \quad \mathbf{TI} = \frac{\mathbf{G} - \mathbf{W}}{\mathbf{G} - \mathbf{U}}$$

2. The arithmetic mean of the index values is determined from a summation of the partial indices and division by their number. This quantity is termed the biological index (**BI**).

$$\text{Formula:} \quad \mathbf{BI} = \frac{1}{n} \left(\sum_{i=1}^{n} \mathbf{TI}_i \right)$$

These two procedures are easily understandable as regards both arrangement and execution. Problems are posed, however, by the question as to how biological age can be calculated from the biological index. Playing an important role in this conjunction is the question of evaluating the individual findings. For this, use has in previous studies been made of empirical clinical values or mathematical weightings. Since problems can be encountered in both cases, the index values of a reference population have been used in the new study to determine an age-dependent reference curve. This can be regarded as a reference quantity of biological age.

RESULTS

The following results are based upon the battery of tests described above. The studies were done in the gerontological laboratory of our department as part of the "Social Gerontology" research project sponsored by the Saxon Academy of Sciences.

It is appropriate at this point to add a general remark. It has frequently been maintained that gerontology is not in a position to develop specific methods and laboratories. The establishment of a functional laboratory for the assessment of biological age proves such

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TABLE 1. BIOLOGICAL INDEX (BI)

Calendar age (years)	BI ♂	BI ♀
20–24	0.209–0.223	0.231–0.246
25–29	0.224–0.239	0.247–0.263
30–34	0.240–0.256	0.264–0.282
35–39	0.257–0.274	0.283–0.302
40–44	0.275–0.293	0.303–0.323
45–49	0.294–0.314	0.324–0.345
50–54	0.315–0.336	0.346–0.370
55–59	0.337–0.360	0.371–0.396
60–64	0.361–0.385	0.397–0.424
65–69	0.380–0.413	0.925–0.453
≥ 70	0.414 (−0.473)	0.494 (−0.520)

a view to be false and could set the trend to establish similar facilities. The plotting of a reference curve required a total of 365 subjects to be included in a randomized study. The results are given in Table 1. The sexual difference is reflected in a parallel displacement which must not be considered an expression of premature aging of the female sex, but corresponds to a specific response of the two sexes to the spectrum of methods used. It is still necessary for the curve to be supplemented in the most advanced age groups.

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DISCUSSION

Our current knowledge and the present results allow the following conclusions to be made:

1. For an assessment of biological age it is necessary to plot a locally and temporally defined reference curve. Such a model must be based upon age-specific, that is, valid parameters.
2. For a comprehensive determination of biological age it is necessary to consider physical, psychical, and social quantities. Quantifiability is a prerequisite of their selection.
3. Calculation of a so-called biological index is possible with corresponding normalization of the test units. The corresponding reference curve can be taken as a base for the calculation of biological age.
4. It is important to note that the recommended battery of tests cannot be substituted for a general checkup and also does not allow the further duration of life to be predicted.
5. The procedure described can be supplemented or varied through the incorporation of additional parameters.
6. Finally, it is necessary to answer the question as to the motivation of such studies.

We believe that biological age determination is of great importance to all areas of gerontology, this being corroborated by the examples given below:

- The method enables expositional *noxae,* that have to be regarded as factors of risk to the course of human life, to be recognized in both their dynamics and importance and, possibly, controlled effectively.
- It is hoped that such methods offer accurate and objectifiable criteria for the verification of forms of geriatric therapy so as to get out of the stage of speculations.

• An assessment of biological age also serves the purpose of measuring the actual functional power and capacity. It is in this particular sense that biological age determination may be considered a basis for the evaluation of human performance capability and functionality. This is true especially of geriatric practice inasmuch as such methods enable the functional capacity of individuals approaching the pensionable age or intending to change their jobs to be measured reliably. However, it will be necessary to examine if, and to what extent, adequate test batteries will have to be developed in order to satisfy specific requirements posed, for example, by occupational circumstances.

The crucial motives for our studies are, therefore, to be found in the requirements of practice.

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ENTEROSORPTION IN PROLONGING OLD ANIMAL LIFESPAN

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Abstract—The effect of repeated courses of enterosorption upon the mean and maximal lifespan and some functional and metabolic indices was determined in 28-month-old Wistar rats. Significant increase of mean and maximal lifespan of old rats was noted at certain regimens of enterosorption. The experimental animals demonstrated less marked age-related structural and ultrastructural changes in the liver, kidneys, myocardium, intestines, pancreas, as compared with control animals. Enterosorption leads to a reduction of pentobarbital-induced sleep, decrease of content of cytochrome P-450, blood cholesterol and triglycerides, cardiac and cerebral tissue cholesterol, total lipids, liver cholesterol and triglycerides. Enterosorption was found to increase the RNA and protein biosynthesis in the liver, kidneys and adrenals of old animals.

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INTRODUCTION

The speculation regarding the possible role of toxic metabolites in the genesis of aging, put forward by I.I. Mechnikov as early as at the beginning of this century, justifies the use of modern sorption methods for influencing the course of aging, for prolonging the lifespan. While considering this problem, a note should be taken of the age-related rise of organism's sensitivity to some toxic agents (Frolkis, 1970). Most of the known experimental methods for lifespan prolongation proved to be effective when used well before the onset of aging. If an assumption is made that the toxic metabolites are essential in the genesis of aging, then the sorption methods should, obviously, be used in late ontogenesis, when these waste products alter quantitatively.

At present, sorption methods are widely used in clinical practice. Quite widespread is the use of enterosorption confined to regular daily intake of 20–80 g of microspheric activated carbons of synthetic origin (Bonatskaya & Zinevich, 1982; Nikolaev *et al.*, 1982; Shcherbitskaya *et al.*, 1982). The main mechanism of enterosorption's therapeutic effect lies in the purification of 6–9 litres of digestive juices, secreted in man daily. In other words, enterosorption may be considered as a version of hemosorption, since the sorption of substances from digestive juices also influences the blood composition. Enterosorption is known to modify the lipid and aminoacid spectrum of the intestinal content, and to

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eliminate toxic and some biologically active substances. Lipid- and cholesterol-eliminating effect of orally taken activated carbons, initially demonstrated in patients with chronic renal insufficiency, established the basis for the use of enterosorption in hypercholesterolemia-related atherosclerosis (Nikolaev & Strelko, 1979). In this context, enterosorption as a possible means of effect upon an aging organism can be recommended.

METHODS

Experiments were performed on 120 male Wistar rats aged 28 months. The hemosorption was performed on carbon sorbent SKN-1k and enterosorption on SKN sorbent (non-coated nitrogen-containing carbon, fraction composition – 0.3–1.0 mm, pore volume measured by benzol – 0.6–0.7 ml/cm³). Animals were fed sorbent-containing diet (1 ml/100 g b.w.) starting from the 28th month of age. The animals were divided in 4 groups according to enterosorption regimen: Group 1–control animals (fed standard diet); Group 2–sorbent administered by 10-day courses at 1-month intervals; Group 3–sorbent administered by 1-month courses at 10-day intervals; Group 4–sorbent administered by one 20-day course. The mean lifespan at 50%, 80% and 100% mortality, as well as the maximal lifespan, were determined. A number of functional, morphological and metabolic indices were estimated in some animals of each group after 5 courses of enterosorption (34–35 months of age) and in animals of Group 4. The myocardium, liver, kidney, lung, brain, pancreas, adrenals, spleen and intestines of 10 animals from each group were used for light microscopical study. All material was fixed in 10% formaline, paraffin embedded sections were stained with hematoxylin-eosin, pycrofuxin. In addition, fragments of each organ were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed in 1% OsO_4 in phosphate buffer (pH 7.4) and embedded in Epon 812. Thin sections were made by LKB III ultramicrotome and stained with uranil acetate, lead citrate and examined in JEM-100B electron microscope.

The indices of the state of lipid metabolism were studied in the blood serum, liver, heart and brain. Extraction of lipids from tissues was made by chloroform-methanol mixture (Folch *et al.*, 1957). Total lipids were determined according to Bragdon (1951), triglycerides according to Carlson (1963), total cholesterol according to Ilka (1962). α-cholesterol was determined after the sedimentation of low and very low density lipoproteins from the serum by $MnCl_2$ in the presence of heparin (Ilka, 1962). The concentrations of the studied substrates in blood serum were expressed in g/l for total lipids and mg% – for the rest. In tissues, the concentrations was expressed in mg/g tissue for all indices.

The content of liver cytochromes P-450 and B_5 were determined in post-mitochondrial supernatant (9,000 g) according to Omura and Sato (1964).

The duration of sodium pentobarbital-induced narcotic sleep (25 mg/kg) was estimated from the moment of "side-position" till the recovery of locomotor reactions.

The intensity of *in vitro* biosynthesis of total RNA and protein was determined in the frontal cortex of large hemispheres, hypothalamus, pituitary, skeletal muscle, left ventricle of the myocardium, adrenals, kidney and liver by the incubation of their sections in the blood serum of the same animal at 37°C and constant bubbling with mixture of O_2 and CO_2 (95:5). After about one-hour preincubation, the labeled precursors of RNA (^{14}C-orotate) and protein (^{3}H-leucine) of 10^2MBq/ml and 10 MBq/ml (final concentration), respectively, were added. After one hour the sections were homogenized in 10% trichloroacetic acid and acid-insoluble material was separated from acid-soluble one by filtration through nitrocellulose membrane filters. Sample radioactivity was measured on scintillation counter "Mark-III."

RESULTS AND DISCUSSION

Hemosorption is known to be an effective means of removing the toxic agents from the blood. In the first series of experiments the study was undertaken on the effect of hemosorption upon the old animal organism. It was found that after 4–6 weeks following the course of hemosorption the old rats developed arterial hypertension. Thus, the initial level of arterial blood pressure in 32-month-old rats made 84.6 ± 6.1 mm Hg, as compared to 118.0 ± 5.4 mm Hg, observed 4–6 weeks following hemosorption. Without conducting special experiments, it is difficult to interpret the mechanism of post-hemosorption hypertension in old rats. Noteworthy are the two facts. First, arterial hypertension develops not instantaneously, but in 4–6 weeks after hemosorption; second, the evolved hypertension is of a sustained character and lasts for several months. It can be assumed, therefore, that the development of arterial hypertension is linked not merely with the sorption of some physiologically active substance, but rather with the slowly evolving regulatory renal and hypothalamic rearrangements.

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All these consequences of hemosorption and its possible complications (Lopukhin & Molodenkov, 1978) have determined our interest in a milder method of body detoxication, that is enterosorption. We also hoped that quite effective could be a long-term sorption of toxic agents, rather than a short-term course, usually practiced in hemosorption.

Another series of experiments (Groups 2 and 3) were made to study the effect of the two enterosorption regimens upon the lifespan of old rats (aged 28 months). In Group 2 enterosorption resulted in the prolongation of both mean and maximal lifespans. In control group the mean lifespan at 50% mortality was 936.6 days, at 80% 972.1 days, at 100% 992.8 days, and at maximal lifespan 1117.5 days, as compared with 977.4, 1022.8, 1054.7, and 1209.5 days, respectively, registered in experimental animals.

The experimental procedure we have chosen required the estimation of changes in the lifespan since the moment of sorbent intake, that is from 28 months of age. Thus, enterosorption resulted in the increase of mean lifespan by 47.3%, 41.4% and 43.7% at 50%, 80% and 100% mortality, respectively; the maximal lifespan increased by 34.4%. In other words, the data obtained indicate that the enterosorption is an effective means of prolonging old animal life. It is noteworthy that the positive effect can be attained only at certain regimens of enterosorption. Thus, the mean lifespan in Group 3 (one-month enterosorption course at 10-day interval) made only 97.9%, 107.3% and 110.3% at 50%, 80% and 100% mortality; the maximal lifespan made 118.9%, as compared with control.

Our special experiments showed that the enterosorption not only alters the animal lifespan, but also influences the rate of onset of age-related structural and metabolic changes in the organism, and the biological age of animals. We have compared the structural and ultrastructural enterosorption-induced changes in Group 2 with those in control animals. In Group 2 we found less marked age-related structural rearrangements and lack of rough pathological changes. Thus, in the heart of experimental animals the diffuse myofibrosis was less marked, and there were no focal sclerotic changes. In contrast to control, the experimental animals showed no contractural and metabolic lesions in the heart. In only one case there was a small focus of fibrosis, located around the blood vessel, while in the coronary vessels and aortal wall no pathological processes have been found.

In kidneys of experimental animals we failed to find sclerosed renal glomeruli, which was the case in control animals. The basal membranes of the glomerular capillaries were not thickened. In the capsules of Shumliansky-Bowman, the sclerotic processes were not marked. The tubuli appeared to be normal, though in one case there was a widening of renal tubuli and filling of their lumen with protein.

In liver, the architectonics of the lobes was normal. Hepatocytes had predominantly light cytoplasm and large nucleus with the fine network of chromatin and large nucleoli. Their cytoplasm contained much less lipid drops, as compared with control. A significant number of bi-nuclear hepatocytes could be seen. The number of Kupfer cells was increased, their cytoplasm had a somewhat basophilic shape, nuclei were distinct, as compared with control. In pancreas, the sclerotic changes were not marked. Both small and large Langerhans islets could be seen. No pathological changes were found in small and large intestines, in lungs and other organs. Thus, enterosorption prevented the development of many age-related structural changes.

Electronmicroscopic examination of the cardiocytes of experimental and control animals revealed moderate swelling of mitochondria with the preservation of the integrity and clarity of the inner membrane and some clarification of mitochondrial matrix (Figure 1). Some mitochondria had destructive changes such as injury of the crystae and avail-

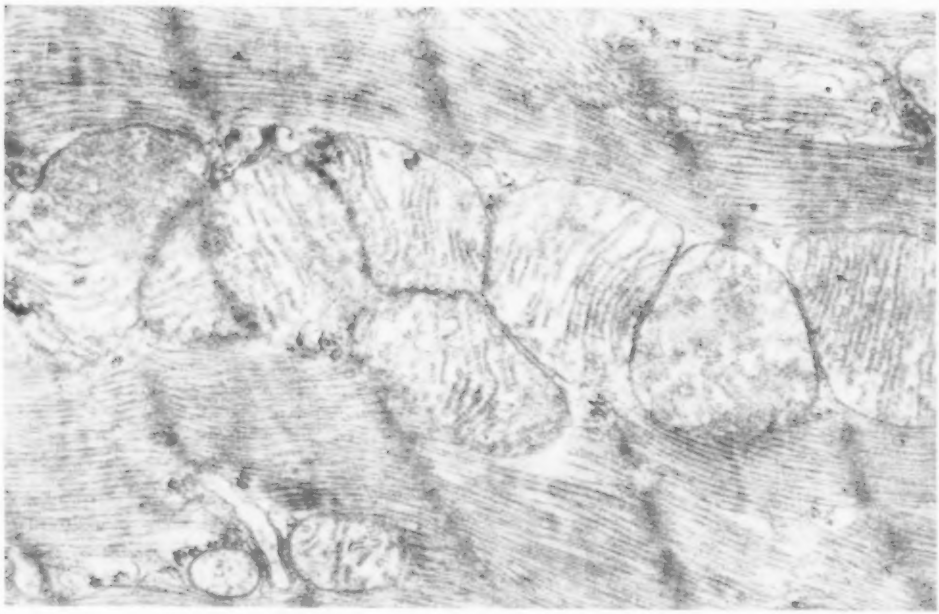

FIG. 1. Electron micrograph of cardiomyocytes of an old rat (Group II) showing insignificant changes in mitochondria (m) and myofibrils (mf) × 22800

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ability of the myelin-like structures. However, the destructive changes of the cardiocytes in Groups 2 and 3 were less marked, as compared with control. The activity of the fibroblast cells was found in the myocardium, that is the swollen cisternae of the rough endoplasmatic reticulum and numerous ribosomes on the surface of the cisternae. Lipofuscin granules were found in the cytoplasm of the connective cells (Figure 2).

Very few lipid granules could be seen in the liver cells in Groups 2 and 3, as compared with control. In addition, the irregular-shaped structures of the lipid origin encircled by osmophilic membrane were noted in the cytoplasm of these cells. The rough endoplasmatic reticulum of the liver cells was well developed and the nuclei contained the diffusive chromatin with large nucleoli (Figure 3).

Thus, light and electronmicroscopic studies showed the marked structural differences between Groups 2 and 3 and control in the liver, kidney and myocardium. The diffusion fibrosis of the organs and tissues was less marked after the course of enterosorption. The injury and destruction of the parenchyma cells were not found in the kidney and myocardium of animals of any experimental group.

The results of the light microscopic study showed no structural changes in the organs and tissues in Group 4 (20-day course of enterosorption).

The duration of narcotic sleep is an accepted index for detoxicating function of the liver. Our experiments revealed that the duration of pentobarbital-induced sleep in control group was 78.75 ± 10.47 min as compared with 49.80 ± 8.94 min in experimental group, that is by 37% less.

The enterosorption produced significant decrease of cytochrome P-450 (by 54.9%), that is 4.81 ± 1.1 nmol/g tissue in control group versus 2.17 ± 0.25 nmol/g tissue after a

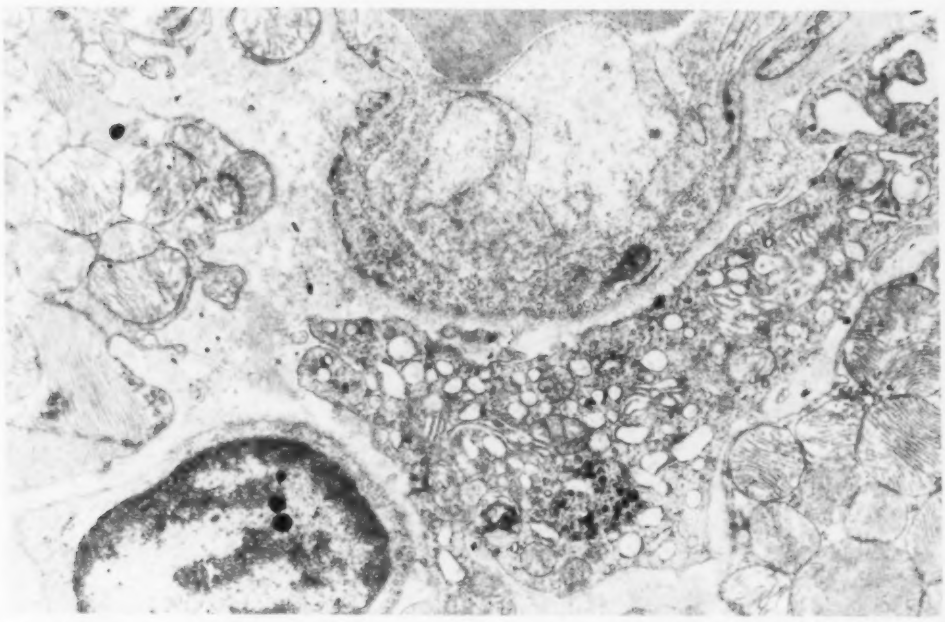

FIG. 2. Ultrastructure of myocardial fibroblast of an old rat (Group II): extended tubules of rough endoplasmatic reticulum (ER) and lipofuscin granules (arrow) in the cytoplasm × 12200

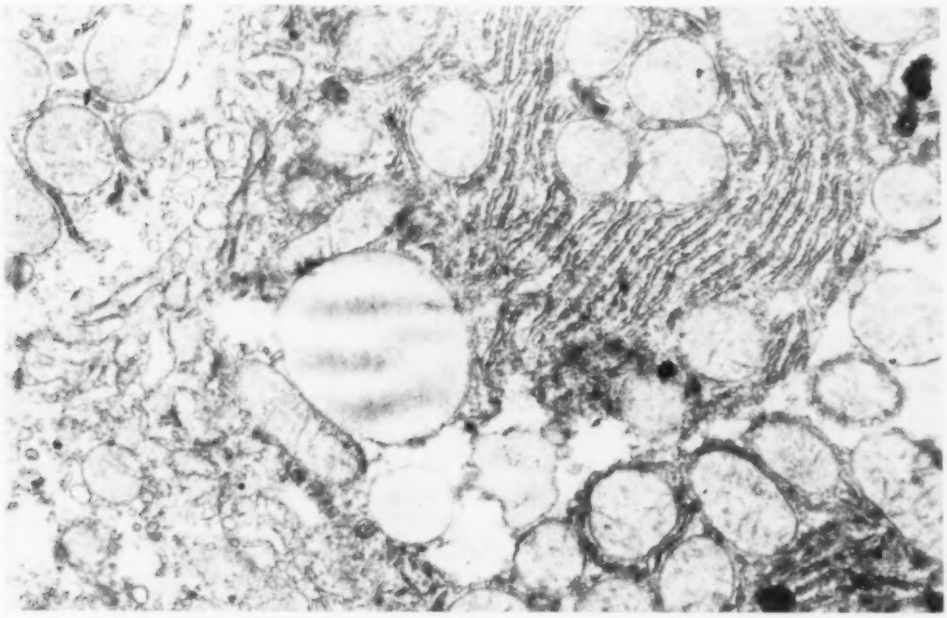

FIG. 3. Electron micrograph of hepatocyte of an old rat (Group II) showing well developed rough endoplasmatic reticulum (ER), normal mitochondria (m) and lipid granules (arrow) × 16000

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20-day course. The content of cytochrome B_5 remained almost the same (11.40 ± 3.26 nmol/g tissue versus 12.23 ± 2.34 nmol/g tissue, $p > 0.2$, respectively). Thus, enterosorption shortened the period of narcotic sleep and decreased the content of cytochrome P-450, a key enzyme of the system of microsomal oxidation in the liver. Usually, the reduction of intensity of microsomal oxidation is accompanied by the prolongation of the narcotic sleep, since in these conditions the narcotic substance has a prolonged effect upon the corresponding nervous centers. In our experiments, the decrease of cytochrome P-450 was, apparently, related with the enterosorption-induced decrease of the concentration of toxic agents, circulating in the blood, which were the substrates of the enzymes of microsomal oxidation. During aging, the sensitivity of the central nervous system to some pharmacological substances, including narcotic ones, increases (Frolkis, 1970). It can be assumed that by influencing the rate of aging the enterosorption decelerates the age-related rise of brain sensitivity to narcotics, thus leading to shortening of pentobarbital-induced sleep in experimental animals, as compared with control.

Another important criterion of the enterosorption efficacy is the state of lipid metabolism. This is conditioned by the role it plays in the processes of aging, in the development of age-related pathology. As is known, during the "enterohepatic circulation" cycle the cholesterol and the main products of its degradation–bile acids–are excreted with bile by the liver and then a large share of them is reabsorbed. And here, in physiological conditions in rats and, possibly, in man (Polyakova, 1981) the cholesterol synthesis and formation of bile acids are coordinated by the negative feed-back mechanism. Therefore, the level of reabsorption of cholesterol and bile acids is essential in maintaining the cholesterol homeostasis in the organism. That is why the enterosorption may be used in an attempt to influence the lipid and cholesterol metabolism in old age.

For this purpose, we have estimated the concentration of total lipids, cholesterol and triglycerides in the blood serum, liver, heart and brain, as well as α-cholesterol in blood serum. Also estimated was the cholesterol atherogeneity coefficient (**C**) (2), which presents the relation of the sum of cholesterol of low density and very low density lipoproteins to cholesterol of high density lipoproteins (α-cholesterol):

$$\mathbf{C} = \frac{\textbf{total cholesterol} - \alpha\textbf{-cholesterol}}{\alpha\textbf{-cholestrol}}$$

We found that the enterosorption resulted in changes in the lipid content in serum and organs. The most significant changes have been found in the liver, where the concentration of total lipids, triglycerides and cholesterol fell by 31.8%, 48% and 28.6%, respectively (Table 1). This effect was marked in continuous enterosorption as well, that is the liver cholesterol and triglycerides fell to 1.78 ± 0.07 mg/g and 12.4 ± 1.36 mg/g, respectively or by 48.6% and 29.2% less than in control group.

Liver plays an important role in the processes of formation of lipids and, primarily, of cholesterol both for own membranes, and for export within very low and low density lipoproteins, as well as in the excretion of cholesterol with bile and its oxidation to bile acids. In view of the above, the decrease of total lipids, triglycerides and cholesterol in the liver may be decisive in changes of lipid metabolism in the whole organism and in the prevention of risk factors for the development of atherosclerosis during aging. In fact, in blood serum we registered significant decrease of total lipids (from 2.99 ± 0.13 to 2.41 ± 0.11 g/l, $p < 0.01$ or by 19.4%) and of concentration of total cholesterol (from

TABLE 1. EFFECT OF ENTEROSORPTION ON INDICES OF LIPID METABOLISM IN ORGANS OF 28-MONTH-OLD RATS (MG/G)

	Liver		*Heart*		*Brain*	
Indices	*Control*	*Experiment*	*Control*	*Experiment*	*Control*	*Experiment*
Total lipids	24.9 ± 2.7	17.0 ± 1.0*	10.8 ± 0.5	9.9 ± 0.5	34.4 ± 1.3	33.9 ± 1.3
Total cholesterol	3.5 ± 0.1	2.5 ± 0.2*	1.4 ± 0.03	1.2 ± 0.03	21.9 ± 0.7	24.0 ± 0.7
Triglycerides	17.5 ± 2.9	9.1 ± 0.7*	4.3 ± 0.4	2.5 ± 0.3*	9.3 ± 0.8	7.7 ± 0.3*

*$p < 0.05$

95.0 ± 5.0 to 79.6 ± 2.8 mg%, $p < 0.02$ or by 16.3%). In 28-month-old experimental animals these indices approximated those of 20-month-old intact rats (2.41 ± 1.0 g/l and 82.0 ± 6.0 mg%, respectively).

Rats are known not to develop spontaneous atherosclerosis (Klimov, 1981), and experimental atherosclerosis is very difficult or even impossible to induce. In rats, a large share of cholesterol is within high density lipoproteins (α-cholesterol). We found that in both control and experimental animals the main amount of cholesterol is within high density lipoprotein fractions (61.0 and 70.3%, respectively). Still, enterosorption resulted not only in the changes of total cholesterol, but also in the increase of α-cholesterol. The cholesterol atherogeneity coefficient decreased 1.5-fold (from 0.64 ± 0.07 in control to 0.42 ± 0.06 in experimental animals). The concentration of triglycerides in the blood remained unaffected, while in the heart and brain it fell significantly ($p < 0.05$) by (40.7% and 17.3%, respectively).

The data presented suggest that the enterosorption-induced changes in lipid content in blood serum and in the studied organs, especially, in liver, are related with changes in enterohepatic circulation. Obviously, the sorbent adsorbs part of cholesterol and bile acids in the intestines, thus eliminating them from the organism. This results in a decreased reabsorption from the intestines. This leads to the enhanced oxidation of cholesterol to bile acids in liver, and both directly and indirectly promotes the decrease of its concentration in blood serum. Such a mechanism of hypolipidemic effect is postulated for cholesteramine, beta-cytosterine and surgery for partial resection of intestinum ilium, that is for effects aimed at the decrease of cholesterol.

The enterosorption-induced decrease of triglyceride content may account for the disturbance of absorption from the intestines of hydrolysis products–lipids and carbohydrates–which determine the rate of lipogenesis in the liver and control the level of blood triglycerides.

The life-prolonging effect, observed against the background of enterosorption may be greatly conditioned by the shifts in plastic provision. That is why the next series of experiments was designed to study the differences in the intensity of protein and total RNA biosynthesis in sections of some organs of the control and experimental animals (Table 2). Based on specific radioactivity of acid-soluble material, we found no significant changes in the uptake of labeled precursors in any of the studied organs, except for the myocardium and skeletal muscle, in which this index slightly decreased. At the same time, the intensity of protein and RNA biosynthesis, estimated by the relative specific radioactivity, increased markedly in the liver, adrenals and less markedly in the kidneys and remained unchanged in the hypothalamus, pituitary, skeletal muscle and myocardium. The relative

TABLE 2. EFFECT OF ENTEROSORPTION UPON THE INTENSITY OF UPTAKE (SPECIFIC RADIOACTIVITY, SR) AND INCORPORATION IN MACROMOLECULES (RELATIVE SPECIFIC RADIOACTIVITY, RSR) OF LABELED PRECURSORS OF RNA AND PROTEIN IN VARIOUS ORGANS OF RATS

	RNA				*Protein*			
	Control		*Enterosorption*		*Control*		*Enterosorption*	
Organ	*SR • 10³*	*RSR • 10³*	*SR • 10⁻³*	*RSR*	*SR • 10⁻³*	*RSR*	*SR • 10⁻³*	*RSR*
Hypothalamus	38 ± 5	1.81 ± 0.33	38 ± 2	1.39 ± 0.11	417 ± 47	0.70 ± 0.06	389 ± 28	0.87 ± 0.14
Pituitary	49 ± 4	15.9 ± 2.3	56 ± 7	15.5 ± 2.5	382 ± 25	11.1 ± 1.5	412 ± 38	10.3 ± 1.5
Adrenals	60 ± 2	1.79 ± 0.16	60 ± 4	2.44 ± 0.31	350 ± 11	2.13 ± 0.24	352 ± 22	4.18 ± 0.44*
Myocardium	78 ± 2	3.05 ± 0.15	70 ± 4	3.17 ± 0.30	406 ± 12	2.78 ± 0.23	370 ± 17	3.09 ± 0.32
Kidneys	74 ± 4	3.54 ± 0.17	70 ± 2	4.25 ± 0.39	368 ± 21	2.56 ± 0.32	346 ± 11	3.00 ± 0.28
Liver	62 ± 5	6.41 ± 1.15	64 ± 5	10.05 ± 1.53	304 ± 15	7.12 ± 1.33	306 ± 22	11.25 ± 1.75

*Significant shifts as compared with control ($p < 0.01$)

specific radioactivity of total RNA and protein in sections of kidneys of experimental animals increased by 17 and 20% as compared with control. In the adrenals and liver, these indices made 59% and 37% and 58% and 57%, respectively. Apparently, the increase of intensity of protein and RNA biosynthesis, which improves the plastic provision of such essential organs as liver, kidneys and adrenals, results in widening the range of some defensive systems of an organism, which, in its turn, leads to lifespan prolongation.

Thus, the data presented suggest that the enterosorption has a potent effect upon an aging organism. This notion can be supported by the enterosorption-induced changes in lifespan prolongation, structural and ultrastructural changes, shifts in protein and RNA biosynthesis and in lipid metabolism, in the content of microsomal enzymes, and so forth. This work substantiates the need for further search for regimens of enterosorption, and combination of it with other effects, aimed at lifespan prolongation. Currently, the question remains whether the described enterosorption effects are related with the sorption of the known or supposed metabolites, or with the changes in the content of some physiologically active substances and subsequent regulatory transformations, or with both. The main thing is that the experiments with enterosorption persuade that altering the humoral environment of an organism may effect the rate of aging, the biological age of animals, and thus their lifespan.

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NEUROTRANSMITTER RECEPTORS IN THE CENTRAL NERVOUS SYSTEM AND AGING: PHARMACOLOGICAL ASPECT (REVIEW)

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Abstract—The present review is concerned with the modern ideas about age-related changes of neurotransmitter receptors in the central nervous system and the possible ways of their pharmacological regulation. Based on the experimental data, attention has been paid to substantiation of the degree of maintenance of the receptor reactions in the CNS during aging in response to various pharmacological manipulations. Finally, the concept about an important role of the disturbances of neurotransmitter processes and their receptor link in the mechanisms of aging of the brain has been postulated.

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NEUROBIOLOGY OF AGING which is essentially concerned with assessment of the role played by age-related changes of the nervous system during aging and analysis of the molecular, structural, and functional mechanisms responsible for the disturbances of its activity has emerged as an important area of modern gerontology most intensively developing in recent years (Frolkis, 1981).

One of the key problems of modern neurobiology of aging is the study on neurotransmitter processes in old age (Pradhan, 1980). This is explained by the circumstance that acquired knowledge about this question helps understand the principal regularities of aging of the central nervous system (CNS) and hence of the entire organism. In other words, these studies are of interest in terms of theoretical and experimental gerontology and at the same time they are of an immediate practical value since an understanding of the pathways of pharmacological regulation of neurotransmission is directly linked with the biochemical interpretation of the means of clinical management of various forms of neuropsychological pathology in old age (Chebotarev, 1983; Dengler, 1982). Until recently however the neurotransmitter processes in the CNS have been predominantly studied at the presynaptic level with special attention being paid to the regulation of biosynthesis and release of the neurotransmitters as well as to the analysis of major enzyme activities in the transmitter metabolism. At the same time such an important link as the receptor postsynaptic apparatus has been studied in less detail. It is the change of the receptor structures that has been recently ascribed a special importance as the leading factor of the CNS aging (Burchinsky, 1983 a; Hirschhorn, 1982; Roth and Hess, 1982). How-

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ever, the progress of research into this area became possible only after the discovery of biochemical mechanisms of neurotransmitter processes in the CNS and the use of molecular pharmacology methods, in particular, the radioligand binding technique. The latter allows insight into the mechanisms of interaction between the major neuropharmacological agents and different receptor structures. It also allows a model of the changes of the receptor apparatus characteristic of various forms of neuropsychological pathology to be developed, thus leading to an explanation of the adaptive-compensatory processes in the CNS at the molecular level.

Neurotransmitter receptor regulation plays an important role in the ontogenesis and development of the adaptive-compensatory reactions of the CNS with particular relevance to synaptogenesis and synaptic modulation. The mechanisms for such regulatory changes can involve either the number of functional receptors, controlled by induction or protein synthesis blockade, or the affinity of the receptors controlled by allosteric conformation, or the effects on the membrane environment of the receptors (ion channel permeability, adenyl cyclase activity, etc.), or the presynaptic auto-receptors (Olsen *et al.*, 1980).

In the present review attempts have been made to analyze the main trends in the experimental investigations of pharmacological receptor regulation in the CNS with aging as the perceptive way for studying the degree of maintenance of adaptive-compensatory reactions and synaptic plasticity of the central neurotransmitter systems and their role in age-related changes of the CNS activity. The studies on age-related changes in the receptor-effector complex and the relationship between post-receptor and functional disturbances are dealt with in detail in the review by Roth and Hess (1982).

DOPAMINE RECEPTORS

First of all, the heterogeneity of age-related changes in the neurotransmitter metabolism should be mentioned as a distinctly established fact, that is they are not totalitarian or one-way directed, but rather emerge only in certain transmitter systems, have varying degrees of pronouncement, and different mechanisms of action (Anisimov, 1979; Meek *et al.*, 1977; Samorajski, 1977; Goldman-Rakis and Brown, 1981; Hirschhorn *et al.*, 1982). The damage of the catecholaminergic neurotransmission and especially of the dopaminergic one which is confined to a general impairment of its activity is also an established finding (Samorajski, 1977; Gottfries, 1981). The principal point of a given problem is not a mere substantiation of the above facts, but rather an explanation of the mechanisms of emergence of the disturbances of the neurotransmitter system activities, the peculiarities of their regulation and adaptation in aging.

Since the dopaminergic neurotransmission is the first which is affected in aging and since the role of dopamine as a neurotransmitter occurring in many brain areas, for example, cortex, subcortical nuclei, hypothalamic-hypophyseal system, and so forth is well-known, one might suggest an important role played by transmitter disturbances in the dysfunction of these CNS parts which are responsible for the provision of the vital organism's functions affected to a certain degree in aging.

To date, the facts about an impairment in aging of the dopamine synthesizing enzymes–tyrosine hydroxylase and dopamine decarboxylase–in different brain structures have been clearly demonstrated (Anisimov, 1979; McGeer, 1971; Pradhan, 1980). Additional reports have described an increased activity of the catechol-destroying enzymes–monoamine oxidase and catechol-O-methyltransferase (Robinson, 1975; Stramentinolo *et al.*, 1977)–as well as weakening of the feedback transmitter uptake (Jones and Finch, 1975). All this in-

disputably points to an impaired dopamine regulatory role in the CNS during aging. However, a true progress in the studies on dopaminergic neurotransmission became only possible owing to the discovery of specific dopamine receptors in the CNS (Kebabian and Greengard, 1971; Kebabian *et al.*, 1972). At the same time, numerous findings that have been recently collected have caused serious reconsideration of the viewpoint about the single dopamine receptor that found its reflection in the gerontological investigations also.

The convincing evidence in favor of the theory of multiple dopamine receptors first formulated by Kebabian and Calne (1979) has been currently obtained both from the analysis of behavioral responses following the administration of dopamine agonists and antagonists and from the study on regulation of the dopamine-dependent adenylate cyclase activity and, in particular, from the direct study on dopamine receptors using the radioligand binding method. Kebabian and Calne (1979) distinguished the D_1 receptors (adenylate cyclase bound) and D_2 receptors (adenylate cyclase unbound). The D_1 receptors are stimulated by micromolar concentrations of dopamine and antagonized by the same concentrations of neuroleptics. The D_2 receptors are also stimulated by micromolar concentrations of dopamine, but antagonized by nanomolar concentrations of neuroleptics (Seeman, 1980). Although there is some progress owing to elucidation of the distribution of the D_1 and D_2 receptors in different CNS areas, the physiological value of each of these dopamine receptors remains unclear. In particular, evidences for the absence of correlation between the blockade of adenylate cyclase activity by neuroleptics and their pharmacological activities can be accounted for by the different sensitivity to neuroleptics in different brain areas (Arushanian, 1982). Using the radioligand binding technique, it has been found that D_1 receptors exist in the hypothalamus, while D_2 receptors occur in the striatum (Leibowitz *et al.*, 1982). Only a single dopamine receptor subtype, D_2 receptors, occurs in the pituitary (Creese *et al.*, 1982). The system of topographically close but biochemically and functionally diverse dopamine receptors indicates the presence of complex system of their regulation and functioning in the same brain areas.

In recent years, the multiple dopamine receptor theory has been further developed. Some authors have clearly distinguished three (Creese *et al.*, 1982) and even four (Seeman, 1980) subtypes of dopamine receptors in relation to the affinity of dopamine agonists and antagonists to each subtype of these receptors. However, incomplete knowledge about the problem, the controversial data and opinions in this area, and lack of analogous investigations in the gerontological aspect make it possible, while analysing age-related changes of the dopamine receptors, to divide them according to the initial classification of Kebabian and Calne only into the two types: D_1 and D_2 receptors.

A detailed experimental investigation of these receptor types allowed identification of specific ligands to each of these types leading, in essence, to the purposeful study of regulatory aspects of interaction between various drugs influencing the dopaminergic neurotransmission that, in fact, marked the beginning of research into the receptor changes as a function of age. As a rule, these ligands are antagonists of the dopamine receptors–neuroleptics–representing different chemical structures. Among such ligands, ^{3}H-cis-flupentixol for D_1 receptors (Hyttel, 1980), ^{3}H-sulpiride (Woodruff *et al.*, 1980) and ^{3}H-spiperone (Madras *et al.*, 1981) for D_2 receptors, and ^{3}H-spiroperidol for D_1 and D_2 receptors (Marchais and Bockaert, 1980) are available.

When passing onto characterization of age-related changes in the dopamine receptors as an important link of the disturbed dopaminergic transmission during aging, one should note that application of the multiple neurotransmitter receptor theory to the CNS aging

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processes allows explains some key questions in relation to age peculiarities of the neurotransmitter metabolism whose solution can only be possible if an account is taken of the receptor structural changes at the molecular level. From this viewpoint, the study on dopamine receptors and their functioning in an old organism is of special interest.

One of the approaches to the solution of this task is analysis of high affinity binding of different radioligands, generally neuroleptics, However, such investigations may overlook the fact that given ligands possess affinity for different types of dopamine receptors thus making interpretation of the obtained results difficult.

The investigation of this problem is currently conducted in two directions: 1) studies on the binding mechanisms for different ligands by dopamine receptors in the intact animals of different ages; and 2) studies on the regulatory-adaptive mechanisms of changes of the given receptors caused by different pharmacological agents, dopamine agonists or antagonists. The latter direction seems to be the most rewarding at present.

Among the markers of dopamine receptors, ^{3}H-spiroperidol, a neuroleptic drug from the butyrophenones, has received the greatest attention. In aging this drug exibits lower binding to dopamine receptors in the striatum, substantia nigra, hypothalamus and cortex. Based on the Scatchard analysis, most investigators reported that this process was linked with a decrease in the number of the receptors without a change in their affinity (Joseph *et al.*, 1978; Severson and Finch, 1980; Thal *et al.*, 1980; Memo *et al.*, 1980), though the evidence exists (Govoni *et al.*, 1978, 1980) indicating predominantly a decrease with aging of the affinity of dopamine receptors to spiroperidol and haloperidol as compared with their number in the striatum and medulla oblongata. In this respect, the following should be added. Spiroperidol, like haloperidol, is not an ideal means for analysis of the specific changes of dopamine receptors because, as it has been shown above, it binds both with D_1 and D_2 receptors. Furthermore, and this is even more important, spiroperidol binds in all brain areas, except the striatum, with serotonin receptors (Creese and Snyder, 1978; Leysen *et al.*, 1978). However, in the striatum spiroperidol blocks only dopamine receptors thus making valid, while using it, only the data obtained in this particular brain area.

Of great interest are the studies involving the specific antagonists of D_2 receptors, for example, spiperone. Misra *et al.* (1980) and Levin *et al.* (1983) documented a decline of the striatal ^{3}H-spiperone binding during aging as a consequence of the reduction of binding sites rather than the affinity indicating the disturbance of the D_2 receptors binding in the striatum, the CNS area where these receptors are prevalent and thereby greatly determine the activity of dopaminergic transmission in this region.

For characterization of the specific changes of D_1 receptors in aging, the investigators have used, instead of the antagonists, an agonist of the dopaminergic neurotransmission, ADTN (^{3}H-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrochloride), a selective agonist of D_1 receptors (Woodruff, 1982). With this agent, they observed a more marked decrease of ADTN binding in the CNS during aging; this decrease being greater than that with spiroperidol in rabbits (Thal *et al.*, 1980), mice (Severson and Finch, 1980), Fisher 344 rats (Hirschhorn *et al.*, 1982), and postmortal human brain (Severson *et al.*, 1982) at the expense of the reduced binding sites.

However, de Blasi *et al.* (1982) have not found any age-related differences in the striatal ^{3}H-ADTN of the Sprague-Dawley rats demonstrating the possible interspecies and intraspecies differences of changes of various dopamine receptor types during aging. As will be shown below, in the last work performed on the same rat strain the analysis of other

neurotransmitter receptors in the CNS has revealed much less age-related differences as compared with other species and strains of the experimental animals.

The findings strongly indicate that in most experimental animals and man the changes in aging predominantly occur in D_1 receptors bound with adenylate cyclase, as compared with D_2 receptors mainly antagonized by spiroperidol.

Hence one can make an important conclusion in the gerontological sense, namely: the unequivocal changes in the D_1 and D_2 receptors within one anatomical area of the brain can be responsible for different functional changes occurring in the CNS during aging. Important in this respect is the use of various dopaminergic substances and the specific ligands for D_1 receptors in the studies on neurotransmission in the hypothalamus, the area of the brain where D_1 receptors prevail. It is quite possible that diversely directed changes in the activity of different hypothalamic centers during aging (Frolkis and Bezrukov, 1979) can be to some extent conditioned by the "imbalance" in the activity of D_1 and D_2 receptors. Moreover, the prevalent impairment of the D_1 receptor function making a greater number here obviously plays a role in it.

In the studies of the postmortal human brain specimens Severson *et al.* (1982) have shown a decrease in the number of both D_1 receptors and D_2 receptors that represents a true age-dependent phenomenon unrelated to concomitant pathology of the CNS. As for the molecular mechanisms underlying the above changes, they require further investigation, though it is already known that an important factor of the receptor changes with aging is an increased microviscosity of the receptor membrane lipid layer. Its changes bring about the increase of sensitivity of the receptor protein to enzyme degradation, a decrease of the reserve receptor pool and, as a consequence, a reduction of their number and adaptive-compensatory possibilities (Samuel, 1982).

One of the most insightful, recently developed approaches to studying the dopamine receptors in aging is directed stimulation or depression of the transmission in the concrete neurotransmitter system. This approach is interesting as it permits a determination of age-related changes in the plasticity and adaptive properties of the receptors that, in turn, are closely linked with the general adaptive-compensatory reactions in the CNS during aging. This approach acts as an indicator of these reactions if we consider the high degree of sensitivity of the receptor apparatus even to a negligible disturbance of the neurotransmission in the CNS (Misra *et al.*, 1981). For this purpose, blockade of the dopaminergic transmission by means of chronic administration of neuroleptics was used. There took place functional rearrangement of the activity of the brain neurotransmitter systems reflecting their plastic properties (Allikmets and Zharkovsky, 1982). The chronic administration of neuroleptics caused the development of hypersensitivity and hence an increased specific receptor binding indicating the adaptive-compensatory possibilities of the dopamine receptors. For this reason, the neuroleptics, such as fluphenazine and haloperidol, are more often used in the gerontological investigations. After termination of chronic fluphenazine administration, both in the young and old rats (less marked in the old animals), there developed hypersensitivity of the postsynaptic dopamine receptors, as was determined by an increased specific binding of ^{3}H-spiperone, a selective blocker of D_2 receptors (Misra and Smith, 1980; Misra *et al.*, 1981). The greater increase of the binding sites with low affinity in young rats was paralleled by an insignificant increase of the receptor numbers with an unchanged affinity in the old animals. Since the increase of the low affinity receptor sites represents primary and main adaptive reaction to the transmission blockade, in old age this primary response appears to be decreased at least for D_2 recep-

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tors. Additionally, aging rats given a single dose of haloperidol, in contrast to a chronic dose, did not show any age-related peculiarities of the dopaminergic transmission (Severson *et al.*, 1981). Because haloperidol blocks both pre- and postsynaptic receptors (Bacopoulos and Roth, 1981), the data obtained by Severson *et al.* (1981) may suggest that a single administration of haloperidol blocks predominantly the presynaptic dopaminergic receptors, while chronic administration blocks the postsynaptic dopaminergic receptors and hence the function of the former remains unchanged during aging, while that of the latter is diminished. This points once again to the heterogeneity of age-related receptor changes even within one neurotransmitter system.

Interesting, no doubt, is an attempt made by Randall *et al.* (1981) to analyze the comparative effects of dopamine agonists and antagonists on the receptors as a function of age. Having grounded the above findings about a significantly reduced hypersensitivity in old age of dopamine receptors in response to chronic administration of the neuroleptics, in particular, haloperidol, the authors have strongly indicated maintenance of the complete development of dopamine receptor hyposensitivity in response to chronic administration of the dopamine agonist, bromocryptine, which is widely used for the treatment of Parkinson's disease. Thus, impaired in old age are only the receptor reactions that require for their development intensive biosynthetic processes, namely, hypersensitivity reactions linked with the biosynthesis of a considerable number of new receptor structures. Simultaneously, maintenance of the complete response to dopaminomimetics makes reasonable the stimulation of the dopaminergic neurotransmission, which is impaired in old age, and opens certain perspectives for these investigations.

α- AND β-ADRENORECEPTORS

The next important link in the disturbance of neurotransmitter processes during aging are changes in α- and β-adrenoreceptors which are located in different CNS areas and in large measure determine higher neuropsychic functions, in particular, the development of depressive states, a quite common pathology in old age (Burchinsky, 1983b).

Age-related changes of α-receptors have been studied inadequately. For instance, de Blasi *et al.* (1982) failed to find any changes in the number of these receptors in the cerebral cortex of old Sprague-Dawley rats. According to Misra *et al.* (1980), Fisher 344 rats showed a reduced number of the binding sites for the selective ligand of α-receptors, WB-4101, in the cerebral cortex. At the same time, these authors (Misra *et al.*, 1981) have shown that chronic administration of fluphenasine to old versus young animals induced a more marked receptor reaction of the hypersensitivity development, both an increase in the number of receptors and their affinity. These data, as opposed to those obtained by the same investigators for dopamine receptors, have indicated the availability of normal compensatory possibilities in α-receptors during pharmacological effects in senescence aimed at preventing the existing reduction of the number of α-receptors in the intact animals.

The changes of β-receptors during aging have been studied in more detail, though these investigations do not give an unequivocal explanation for them. For instance, in the intact rats of different strains use of the ligand ^{3}H-dihydroalprenolol (DHA) resulted in a decrease of the number of β-receptors without a change of their affinity in the cerebral cortex (Misra *et al.*, 1980; de Blasi *et al.*, 1982). More thorough investigations have revealed the presence of two populations of β-receptors in the CNS, that is high and low affinity receptors. In old age, there occurs a decrease in the number of only high affinity receptors without changing their proper affinity in the cerebellum and brainstem (Maggi *et al.*,

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1979) and in the cortex (Piantanelli *et al.*, 1981). However, the evidences exist which differ from those reported here. According to Aksentsev *et al.* (1982), in rats (strain is not indicated) there is an increase in the number of β-receptors of the CNS during aging parallel to a decrease of their affinity, as well as the disturbance of their conformational mobility. Given that the authors of this work have not distinguished between various β-receptors populations and that use was made of the whole brain specimens, the evidences for the reduced general affinity of β-receptors can be explained by a decrease in the number of their high affinity population, as has been shown by the above data, but an increase in the total number of β-receptors in the whole brain can be caused either by species differences or by some unknown regional peculiarities of the distribution of these receptors in old age.

Quite interesting are the data obtained with pharmacological effects on β-receptors during aging. So, Greenberg and Weiss (1979) reported absence in old age of the development of hypersensitivity reaction by β-receptors in response to reserpine administration. At the same time, they observed maintenance of the ability to react by reduced receptor binding in response to desipramine, an antidepressant, inhibiting the feedback noradrenaline uptake and hence acting adrenomimetically thus decreasing the number of receptors without an apparent change of their affinity. These data well correlate with the analogous findings of Randall *et al.* (1981) in D_2 receptors and may suggest good maintenance of the reactions of catecholamine receptors in response to the stimulation of dopamine- and adrenergic transmission. At the same time, Misra *et al.* (1981) who used fluphenasine have reported the development in old animals of marked hypersensitivity of β-receptors in response to blockade of the adrenergic transmission, that is a considerable increase in the number of β-receptors with much lower affinity, as compared with the young animals. The discrepancy with the results obtained by Greenberg and Weiss (1979) can be easily explained by various courses of drug administration. Because reserpine was administered for three days in the first work and fluphenasine was administered for eight weeks in the second work, it is likely that it is only after a sufficiently prolonged course of administration of the drug, blocker of the catecholaminergic neurotransmission, that old β-receptors began to develop hypersensitivity. However, the differences of mechanisms of action in reserpine and fluphenasine as well as different reaction of β-receptors in old animals to pre- and postsynaptic effects cannot be ruled out.

In conclusion, the analysis of age-related changes in dopamine, α- and β-adrenoreceptors has shown that their reaction to neurotransmission blockade is generally preserved, though much impaired, as the complete response of the old animal requires larger doses and more prolonged course of drug administration. At the same time, the reaction in old age to the stimulation of catecholamine receptors is complete enough and this allows optimistic evaluation of the use of dopamine and adrenomimetics in various forms of senile pathology, and in particular, the depressive states and Parkinson's disease.

SEROTONINE RECEPTORS

Important, though little studied, is the role of age-related changes of the serotonine receptors in the CNS. In general, the serotonine metabolism in aging has been studied inadequately. Evidence exists about its unaltered content in the CNS during aging, as a whole, an increase of its concentration in some regions of the brain (Timiras *et al.*, 1982) and a decreased level of serotonine in combination with an impaired tryptophan hydroxylase activity (Meek *et al.*, 1977). Feedback uptake of serotonine is unchanged with age

(Jones and Finch, 1975). The most exhaustive studies on changes of serotonine receptors in aging have been performed by Shih and Young (1978). They found two types of binding sites in the human brain: with high and low affinity for serotonine. With advancing age, however, there is only one type of receptors, namely, with very low affinity, that is but slightly made up for by an increased number of their receptors. The authors consider the above receptor changes as an important link in the impairment of serotoninergic transmission during aging. However, de Blasi *et al.* (1982) failed to observe any age-related changes of serotonine receptors in the Sprague-Dawley rats. In general, the question about age-related changes in serotonine receptors, especially with pharmacological manipulation, remains to be further investigated.

M-CHOLINORECEPTORS

The next important section of age-related changes in the neurotransmitter processes with aging is the central cholinergic transmission dysfunction. The greatest density of M-cholinoreceptors in the CNS is observed in the extrapyramidal system (Yamamura and Snyder, 1974), and in the cortex that determines the role of acetylcholine in the activity of both subcortical and cortical centers. The functional disruption observed in cholinergic mechanisms of aged subjects is partially responsible for the loss of memory, perception and cognitive processes impaired by age (Bartus, 1980), development of senile dementia of the Altzheimer type (Corkin, 1981), as well as the appearance of complications after chronic administration of neuroleptics and motor dysfunction in Parkinson's disease. Important here are presynaptic disturbances of the cholinergic receptors and, in particular, an impairment of the choline acetylase and acetyl cholinesterase activities, that is enzymes of biosynthesis and hydrolysis of acetylcholine (Vijayan, 1977; Rama Sastry *et al.*, 1983), and slowing of the acetylcholine biosynthesis (Gibson *et al.*, 1981).

The studies on age-related changes in the M-cholinoreceptors of the CNS performed in recent years allow to have a new approach to this problem. Using the specific radioligand for M-cholinoreceptors, ^{3}H-quinuclidinyl benzilate (QNB), some investigators (Freund, 1980; Kubanis *et al.*, 1982) could establish a decrease of M-cholinoreceptors in the whole brain without a significant change in their affinity, that was associated with the decrease in biosynthesis receptors. Further analysis of age-related characteristics of the two M-receptor populations, high and low affinity, has shown their parallel decrease in the hippocampus, again without significant changes in the affinity proper of the individual receptors (Nordberg and Winblad, 1981; Briggs *et al.*, 1982) thus making these receptors different from the adreno- and serotonine receptors.

The characteristic features of M-cholinergic responsiveness in aging have been studied using various pharmacological agents. Old rats injected with the cholinergic receptor-blocker scopolamine exhibited amnesia much earlier than young animals (Bartus, 1980). At the same time, the cholinomimetics currently used in combination with nootropic drugs for therapy of the patients suffering from Altzheimer's disease proved quite effective. In the differentiated studies on various cholinomimetics an interesting regularity has been established: the aged animals exhibited the greatest memory improvement with the direct M-cholinomimetic arecoline, lesser improvement with the cholinesterase inhibitor physostigmine, and the least memory loss improvement was observed with the source of acetylcholine biosynthesis choline (Bartus *et al.*, 1980). The authors concluded from this investigation that the more direct action a pharmacological agent has on the M-cholinoreceptors, the more effectively it normalizes the senile memory losses. The latter under-

lines once again the leading role of receptor changes in the CNS aging and the onset of pathology mediated through the cholinergic mechanisms (Lippa *et al.*, 1980).

GABA RECEPTORS

In recent years, studies have been extensively undertaken on GABA receptors aimed at the elucidation of the role played by the receptor link in the physiological mechanisms of GABA action as an inhibitory transmitter in the central nervous system (Enna, 1981). At the same time, age-related characteristics of these receptors have been studies inadequately. Studies involving ^{3}H-GABA and ^{3}H-muscimol agonist of GABA have revealed both a decrease in the number of GABA receptors (Govoni *et al.*, 1980; Calderini *et al.*, 1981; de Blasi *et al.*, 1982), and absence of any of their changes (Maggi *et al.*, 1979; Lippa *et al.*, 1981; Calderini and Toffano, 1982) in various areas of the brain of different species and strains of the experimental animals.

All the investigators have found a decrease in the number of GABA receptors only in the hippocampus and lack of age changes in them in the cortex. The data were quite contradictory for the cerebellum, subcortical nuclei and medulla oblongate. Lack of studies on the GABA receptors as a function of age with a use of various pharmacological agents as a regulatory factor has not permitted until now to make any grounded conclusions about the direction and mechanisms involved in age-related changes of these receptors and their role in CNS aging.

Of special interest are age-related changes of those CNS receptors whose entity and physiological role have been relatively recently discovered and which are now being extensively studied. They include benzodiazepine and opiate receptors.

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BENZODIAZEPINE RECEPTORS

The mechanisms of action of the tranquillizers of the benzodiazepine row, including those which are used in geriatrics diazepam and its derivatives, for a long time remained unclear. The situation improved thanks to the discovery of specific receptors for benzodiazepines which are anatomically and functionally closely linked with GABA receptors in various CNS areas (Braestrup and Squires, 1977; Mohler and Okada, 1977). Benzodiazepines, on binding with specific receptors through a number of reactions, alter the conformation and potentiate the affinity of GABA receptors of GABA resulting in an enhanced inhibitory neurotransmission and marked tranquillizing effect.

An increased sensitivity to benzodiazepines in aging manifesting in a more marked tranquillizing effect with lower doses (Epstein, 1978) is a well established fact, but it has not been explained satisfactorily as yet. To a certain extent, it may be connected with the delayed kinetics of these drugs in old age (Ochs *et al.*, 1981). Studies on the changes in benzodiazepine receptors during aging allow to have a more thorough insight into this problem. The data from all studies on age-related changes in the benzodiazepine receptors are very contradictory. For instance, in the investigations by Pedigo *et al.*, (1981) the senescent Fisher 344 rats do not show any changes in high affinity binding of ^{3}H-flunitrazepam and ^{3}H-diazepam, respectively, in the cortex, cerebellum, and hippocampus which is consistent with the absence of changes in the allosteric interaction between the benzodiazepine and GABA receptors. If Pedigo *et al.* considered that the major cause of increased age-related sensitivity to benzodiazepines is an altered pharmacokinetics, Tsang *et al.* defined it either as the disruption of the receptor-effector complex or change of these receptors in

other, not yet studied brain regions. However, the studies on benzodiazepine binding in a whole brain did not reveal any differences between old and young mice (Heusner and Bossman, 1981). At the same time, quite different results were obtained in the Sprague-Dawley rats. Memo *et al.* (1981) reported an increased specific binding of ^{3}H-diazepam at the expense of increased number of receptors without a change of their affinity in the cortex, cerebellum and hippocampus. They considered an increased number of benzodiazepine receptors along with the kinetic peculiarities to be the major cause of the increased sensitivity to them in aging. Parallel to them, de Blasi *et al.* (1982) showed in the same rat strain a decrease in the number of these receptors in the hippocampus and cerebellum during aging. A more detailed study on age-related changes of the benzodiazepine binding was undertaken by Calderini *et al.* (1981) and Calderini and Toffano (1982). According to their data, an increase of ^{3}H-diazepam binding in senescent Sprague-Dawley rats was observed in the cerebellum, hippocampus and striatum, but not in the cerebral cortex, that is again consistent with an increase in the binding sites. Interestingly, the binding of ^{3}H-β-carboline-3-carboxylate, a putative endogenous ligand that interacts selectively with benzodiazepine receptors, is in the reversed sequence, greater in the hippocampus and lesser in the cerebellum. The authors reported that ^{3}H-diazepam induced a greater activation in ^{3}H-GABA binding in the older animals, whereas GABA increased ^{3}H-diazepam binding much less in the older group. These data can be interpreted as being a functional correlate of the binding sites, a decrease of GABA receptors and an increase of the benzodiazepine receptors in old age. Consequently, it can be noted that apart from the quantitative changes, the qualitative functional interactions in the GABA-benzodiazepine complex also undergo a change.

In conclusion, there is much confusion and misunderstanding in the studies on benzodiazepine receptors as a function of age, and, possibly, intra- and inter-species specific differences of the receptor binding in different experimental animals play no less important role in it. Special studies involving a parallel use of different species with a detailed analysis of benzodiazepine binding in the central nervous system are needed.

OPIATE RECEPTORS

Although there has appeared an increasingly large body of research data on the opiate receptors in recent years, this problem as a function of age still remains unelaborated. However, even the first investigations have put forward many interesting questions. One of them concerns the different responsiveness of the opiate receptors observed in aging males and females. For example, the male rats showed a reduced specific binding of ^{3}H-dihydromorphine at the expense of a decreased receptor number and an increased affinity in the cortex and striatum, that is the brain areas which are most rich in dopamine (Jensen *et al.*, 1980). One of the possible explanations of this fact can be a close anatomical relationship between the dopaminergic and opiate neurotransmitter systems. Since the majority of opiate receptors are located in the above brain areas presynaptically (Kuschinsky, 1981), the opiate receptors themselves are not likely to be disturbed in the aged males, and their decreased number is linked with the primary dysfunction and reduction of the dopaminergic neurons, considering the normal compensatory reaction to an impaired binding, that is rise of the affinity of the remaining receptors in old rats. Remarkably, these findings agree with the data obtained by the same authors about direct correlation between the binding of another opiate receptor ligand ^{3}H-etorphine in old

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male rats and their impaired behavioral reactions and responses to stress manipulation mediated through the dopaminergic mechanisms. Indirectly, this has been proved by Chan and Lai (1982) demonstrating a decrease in the analgesic effect of morphine in old male rats which is to a large extent mediated through the dopamine- and cholinergic neurotransmitter systems.

At the same time, a decrease in the number of opiate receptors was observed in the thalamus, midbrain and cortex, but not in the striatum, of the aged female rats, this decrease being more marked in the females as compared with the males (Messing *et al.*, 1980). The receptor affinity in the aged females was "intermediate," as opposed to young rats having two types of the opiate receptors–high and low affinity–to binding sites for ^{3}H-dihydromorphine; that is the compensatory increase of the affinity was not observed in the females that was also supported by the data of Hess *et al.* (1981). The reason for the change in opiate receptors in old female rats can possibly be explained by the changes occurring in the hormonal functions mediated through opiate receptors and impaired with aging (Messing *et al.*, 1981).

CONCLUSION

To sum up, it should be noted that studies on the age-related changes in the neurotransmitter receptors now represent one of the most important gerontological problems and one of the most complex in terms of interpretation of the obtained results. Use by the different groups of investigators of different methodic approaches, species and strains of the experimental animals, and lack of the uniform requirements to their age limits make analysis of the great bulk of evidences difficult. It should be also stressed that altered receptors of the intact old animals do not necessarily indicate the dysfunction mediated via these receptors. Only studies of the receptor apparatus using various pharmacological agents would allow to judge about the maintenance of the adaptive-compensatory reactions in the central neurotransmitter systems and hence the validity of functions linked with these systems.

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CYTOCHEMICAL INTERACTION OF NUCLEOLUS AND CYTOPLASM IN THE PURKINJE CELLS OF SENILE WHITE RATS UNDER THE INFLUENCE OF CENTROPHENOXINE

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Abstract—Senile white rats were treated with centrophenoxine at a dosage of 100 mg/Kg body weight/day for 60 days intraperitoneally. Sections of variously fixed and embedded cerebella were studied cytochemically to note the effect of the drug on the senile Purkinje neurons. The nucleolus was found to be hyper-active, as evidenced by the processes of budding and extrusion. A frank regeneration of the Nissl patches along with an increase in α-esterase and decrease in the activity of acid phosphatase and simple esterase was noted in the Purkinje cells after 60 days' treatment. It is suggested that the drug exerts its positive effects by regenerating the general cytoplasm and by revitalizing the nucleocytoplasmic interactions in the senile Purkinje cells.

INTRODUCTION

CENTROPHENOXINE is a synthetic hydrochloride of dimethylaminoethyl-p-chlorophenoxy acetate. Thuiller *et al.* (1959) considered it to be a neuroregulator and they reported that the drug, which is an ester, retards also the aging, yellowing and falling of leaves. Gerontologists became interested in this drug as soon as Nandy and Bourne (1966) and Nandy (1968) reported that it possessed lipofuscinolytic activity. The influence of the drug on the biological process of aging was subsequently investigated at various laboratories, including our own. The drug has not only been established as an effective lipofuscinolytic agent, it is now known also to increase the total life-span of the fruit-flies and mice (Hoschild, 1973). Not only this, the drug also influences the metabolism of the neurons, myocardium and liver. In our studies being presented in this report, frank nucleo-cytoplasmic interactions in the neurons have been recorded under the influence of centrophenoxine.

The nucleolus is a dynamic cell organelle and its composition and synthetic processes change in response to different metabolic states of the cell. The nucleolus is mainly concerned with the synthesis of ribosomal RNA. Caspersson (1950), Brachet (1957) and Nilsson and Leick (1970) have correlated the structural and functional aspects of the nucleolus. They could also relate nucleolar size and RNA content with protein synthesis.

LaVelle and LaVelle (1975) in axotomised neurons, and Manocha and Sharma (1977, 1978) and Sharma and Manocha (1978) in the neurons of protein-deprived squirrel monkeys reported that the nucleolus undergoes a series of changes in response to cytoplasmic chromatolysis. According to these authors, the greater the chromatolytic changes in the cytoplasm the more pronounced the nucleolar synthetic activity.

Though much has been said about the anti-aging activities of centrophenoxine, nothing has been reported on its effects on the nucleolar activity of the senile brain cells. The present report deals with the cytoplasmic interaction of the nucleolus and cytoplasm during the treatment of experimental animals with centrophenoxine.

MATERIALS AND METHODS

Centrophenoxine at a dosage of 100 mg/kg body weight/day was administered to a total of 24 senile female white rats for 60 days, intraperitoneally. A constant room temperature of 25 ± 5 °C and spring light conditions were strictly maintained. Four rats were killed every tenth day and their cerebella quickly removed and fixed variously for histochemical studies. Six senile females served as controls and a group of 6 young females (4 to 6 months old) was used as an additional control.

Tissue sections of the variously fixed material embedded in paraffin/gelatin were used for histological and histochemical studies. Formaldehyde-fixed unpostchromed and postchromed gelatin as well as paraffin sections were preferred for the histochemical study of lipids. Formaldehyde-, Bouin-, Carnoy- and Zenker-fixed paraffin sections were employed for the study of carbohydrates, proteins, nucleic acids and various lipopigments.

For general histology, the haematoxylin eosin and Masson's trichrome techniques were used. The toluidine blue method, though meant for metachromasy, was found quite helpful in clearing the general histology. For histochemical studies, Sudan black B, Sudan III and IV, oil red O, Nile blue sulphate and acid haematein, accompanied by pyridine control, were used for the demonstration of lipids. For carbohydrates containing 1,2-glycol groups, periodic acid-Schiff, followed by acetylation and KOH reversal, was applied. Methyl green-pyronin G was tried for nucleic acids. General protein content was studied by employing mercury bromphenol blue, while the amino acids like tyrosine, tryptophan and histidine were demonstrated by using coupled tetrazonium reaction accompanied by its various blocking techniques, including benzyolation. The Millon and the Sakaguchi methods were preferred for the detection of tyrosine and arginin, respectively. For the various pigments the long Ziehl-Neelsen, the chrome-alum haematoxylin and the Schmorl's techniques were used on formaldehyde-fixed gelatin/paraffin sections. Methods for acid phosphatase, non-specific simple esterases and α-esterases were used on cold acetone/cold formalin-fixed gelatin/paraffin sections. (Details of the various methods as given in Pearse's book of Histochemistry)

RESULTS

Young control group

In the young control group animals, the nucleolar activity in terms of increased size, budding and active migration of material into the cytoplasm could be observed only occasionally in the Purkinje cells; the nucleolus mostly occupied its position in the center of the nucleus. The size of the nucleus and nucleolus presented no significant change in cells with or without nucleolar budding. The cytoplasm did not also present any chromatolytic changes. The perikarya of these cells were packed with Nissl patches (Figure 1). The cytoplasm also presented diastase-digestible PAS-positive particulates. A difused enzyme activity was observed as far as acid phosphatase and simple esterases are concerned. α-esterase activity was, however, greater compared with that in the old rats. For further details, see Table 1.

Old control group

Most of the Purkinje cells belonging to the old control group animals presented degenerative changes by way of chromatolysis, pyroninophobe vacuolar spaces in both

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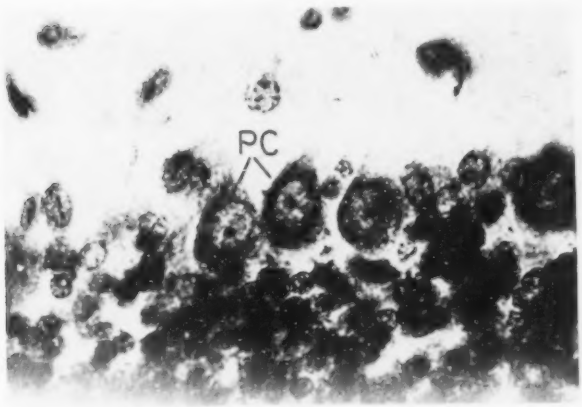

FIG. 1. Tuluidine blue preparation of the Purkinje cell layer from a young control animal showing Purkinje cells (PC) with a heavy package of Nissl patches. (100 × 7×)

cytoplasm and nucleus, as well as lack of Nissl patches in the perikarya (Figures 2,3 & 4). The nucleoli of these cells appeared to be somewhat bigger than those of the Purkinje cells of the young control group. A few neurons also exhibited the process of budding in their nuclei. Even atrophy could be observed in some. The perikarya as well as the nuclei of such neurons were highly swollen, with very little cytoplasm in the latter (Figure 5). A strong acid phosphatase activity and a moderate to intense simple esterase activity was noted in almost all cells of this age group. However, the activity of α-esterase was mild. The cells presented small to huge deposits of lipofuscin pigment at the base of the principal dendrites. Details of histochemical nature of the nucleolus, its buds and cytoplasm are summarised in Table 1.

Experimental group

On treatment with centrophenoxine, the nucleolar activity in the experimental animals was enhanced. The size of the nucleolus increased 2 to 3 times the original one. Almost all Purkinje cells presented chromatolysis and pycnosis—changes that appeared with age as evidenced from a comparison of the old and young control group preparations. The nucleoli in these cells were observed giving out droplets, buds or extrusions (Figures 6–9). The histochemical properties of such nucleoli, their buds as well as the cytoplasm have been tabulated in Table 1.

The buds were usually in the form of spikes or nodules or in the form of granules of different sizes and shapes. The size and number of buddings of the nucleolus seemed to depend totally on the degree of chromatolytic changes in the cytoplasm. This hyper-activity of the nucleoli continued until 40 days of treatment, and by the 60th day the cytoplasm of most of the cells presented enough package of Nissl patches; only a few cells presented pycnotic changes. There was also a significant increase in the total protein content of the Purkinje cells (Figure 10). The nucleoli, by the end of the experiment, occupied the central position.

The cells, on treatment, gradually regenerated their Nissl patches. The process of regeneration was at its peak by the 40th day of treatment. The perinuclear cytoplasm

TABLE 1. HISTOCHEMICAL REACTIONS OF THE NUCLEOLUS, NUCLEOLAR BUDS, SECRETION PRODUCTS IN THE CYTOPLASM AND CYTOPLASM OF THE PURKINJE CELLS.

	Old control			*20 days treated*				*40 days treated*			
Reaction	*Cytoplasm*	*Nucleolus*	*Nucleolar buds*	*Cytoplasm*	*Nucleolus*	*Nucleolar buds*	*Secretion product in the cytoplasm.*	*Cytoplasm*	*Nucleolus*	*Nucleolar buds*	*Secretion product in the cytoplasm.*
Haemotoxylin-eosin	±	2+	2+	±/+	3+	3+	±/+	±/+	3+	3+	±/+
Masson trichrome	+/2+	2+	2+	+	3+	3+	±/+	+/2+	3+	3+	±/+
Sudan III & IV	+/2+	−	−	+/2+	−	−	±	+/2+	−	−	±/+
Sudan black B	+/2+	−	−	+/2+	−	−	±	+/2+	−	−	±/+
Nile blue sulphate	+	−	−	2+	−	−	±	2+	±	±	±/+
Acid Haematin (AH)	+	−	−	+	−	±	±	2+	±	±	±/+
AH after pyridine extraction	−	−	−	−	−	−	−	−	−	−	−
Periodic Acid-Schiff (PAS)	+	2+	2+	+	2+	2+	+	+/2+	2+	2+	+
PAS after acetylation	−/±	−	−	−/±	−	−	−	−	−	−	−
Acetylated PAS after KOH reversal	+	2+	2+	+	2+	2+	−	+	2+	2+	±/+
Toluidine blue	±/+	2+	2+	+/2+	2+	2+	+	2+	2+/3+	2+/3+	+
Methyl green pyronin G (MG/PG)	+/2+	2+	2+	+/2+	3+	3+	+/2+	3+	3+	3+	+/2+
MG/PG after salivary ribonuclease digestion	−	−	−	−	−	−	−	−	−	−	−
Mercury Bromphenol blue	±/+	+	+	±/+	+	+/2+	+	+/2+	+	+/2+	+
Million	−/±	−	−	−/±	−	−	−	±/+	−	−	−
Sakaguchi	±	±	±	±	±	±	−	±	±	±	−
Coupled tetrazonium	±	±	±	±/+	±	±	±	+	±	±	±
Acid phosphotase	+/2+	−	−	+/2+	−	−	−	±	−	−	−
Simple esterases	+/2+	−	−	+/2+	−	−	−	±	−	−	−
α-esterase	+	−	−	+	−	−	−	+/2+	−	−	−

Abbreviations: − = negative; ± = negative in some parts and weak positive in others; + = Weak positive; 2+ = Strongly positive; 3+ = Intensely positive.

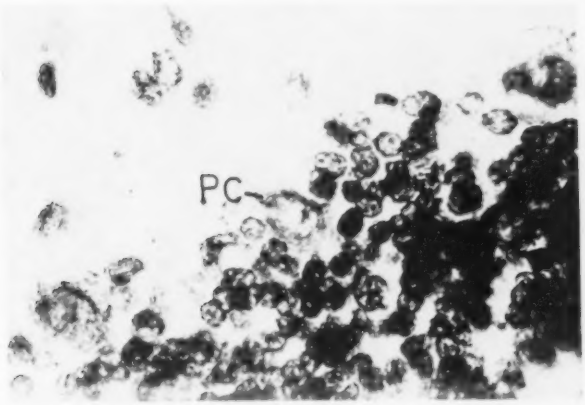

FIG. 2. Toluidine blue preparation of an old control animal showing Purkinje cells (PC) with sparsely distributed Nissl patches. (100 × 7×)

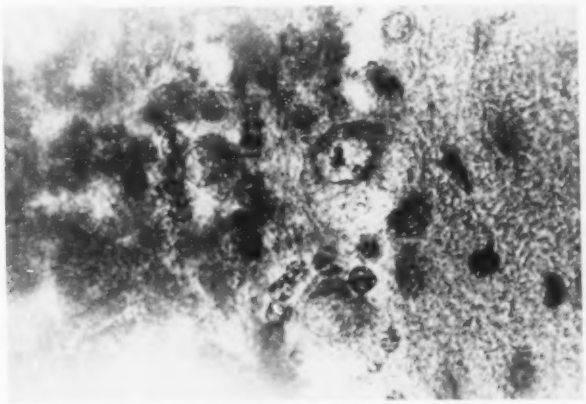

FIG. 3. Haemotoxylin-eosin preparation of an old control animal showing chromatolytic vacuolization and enlargement of the nuclei and nucleoli of Purkinje cells. Note the budding process going on in the nucleoli. (100 × 7×)

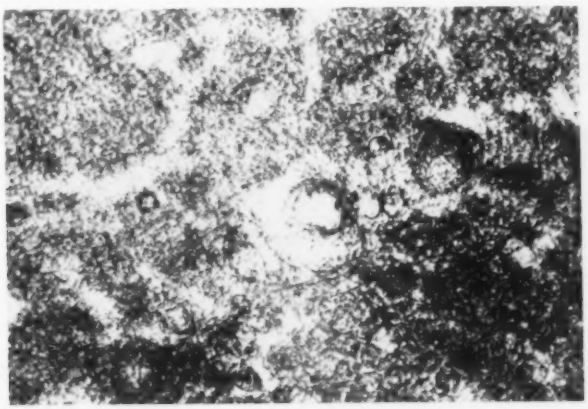

FIG. 4. Mercury bromphenol preparation of a Purkinje cell from an old control animal depicting a negative cytoplasm and nucleolplasm. Branches of dendrites show sparsely distributed protein particles. (100 × 7×)

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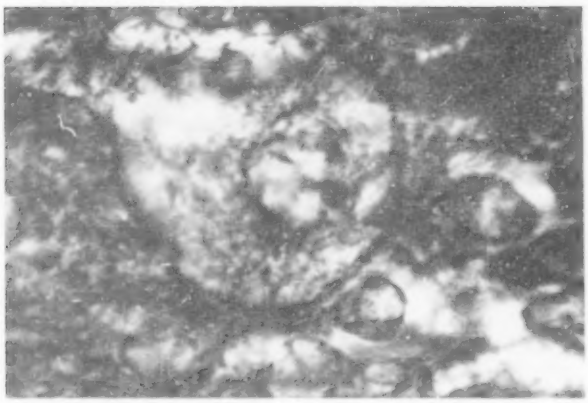

FIG. 5. Mercury bromphenol blue preparation of a Purkinje cell from an old control animal showing swelling of the cell along with chromatolysis and pycnosis. The nucleolus with a bud lies in close vicinity of the nuclear membrane. (100 × 10×)

mostly contained the nuclear secretion product. Sometimes the buds, recently released to the cytoplasm through extrusion, could be recorded and these extrusion products presented almost the same or a little faint cytochemical characteristics. The extrusion products seemed to be totally lost in the cytoplasm after a little while.

Whereas the senile rats presented very little PAS-positive and diastase-digestible glycogen content, the young and treated animals presented enough of these substances. However, the amount of diastase-resistant PAS-positive particles was almost nil as compared to the senile cells. Whatever little lipofuscin pigment accumulated at the base of the principal dendrites of these cells, disappeared totally by the 60th day of treatment.

A reduction in the two lysosomal enzymes, acid phosphatase and simple esterases, was noted. Concomitantly, an increased activity of α-esterase was also observed in the general cytoplasm of the treated cells.

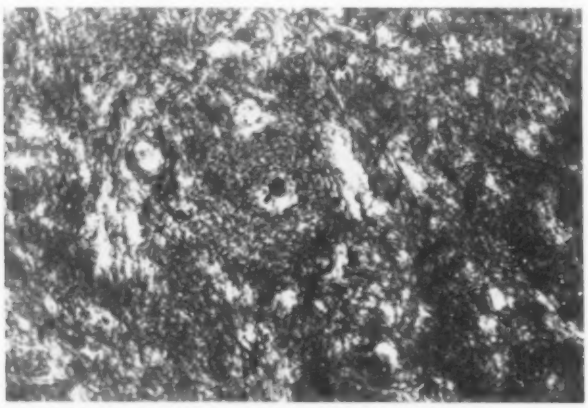

FIG. 6. Toluidine blue preparation of a Purkinje cell from a 40-day treated animal showing a nucleolar bud. Note frank regeneration of the Nissl patches. (100 × 7×)

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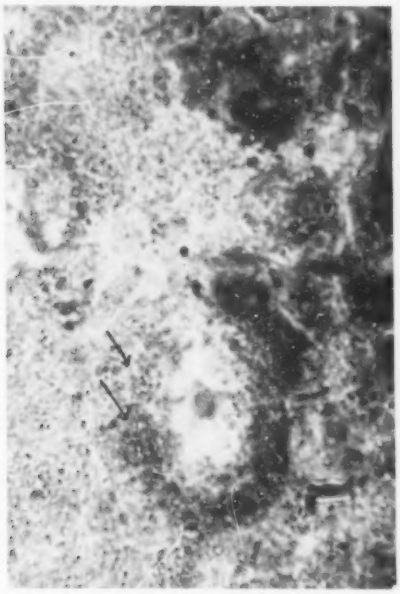

FIG. 7. Methyl green-pyronin G preparation of a 40-day treated animal showing a Purkinje cell exhibiting pyroninophobe vacuolar spaces in the nucleoplasm and the nucleolar extrusions. Note, a mildly stained area (arrow) indicative of a site of cytoplasmic regeneration after chromatolysis. (100 × 10×)

FIG. 8. Ziehl-Neelsen preparation of a Purkinje cell displaying an enhanced budding activity of the nucleolus. Also note pycnotic changes in the nucleoplasm. (100 × 7×)

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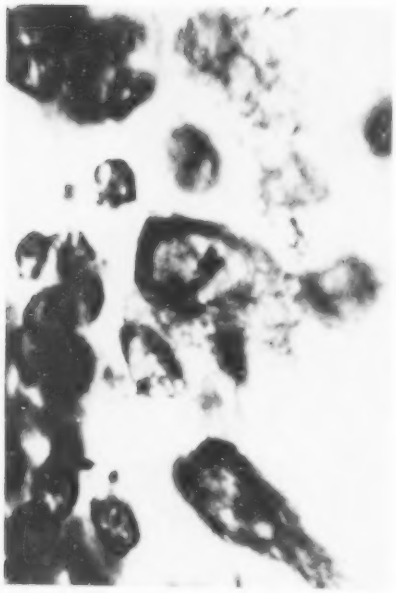

FIG. 9. Toluidine blue preparation of a Purkinje cell of 20-day treated animal presenting a chromatolyzed perikaryon and a nucleolus giving out buds. (100 × 7×)

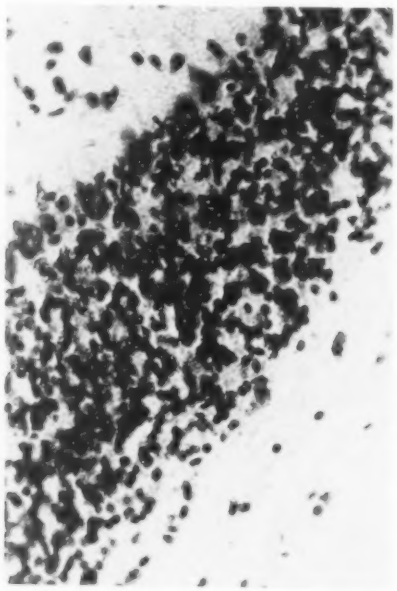

FIG. 10. Toluidine blue preparation of a Purkinje cell of a 60-day treated animal displaying an increase in the volume of Nissl patches in the cytoplasm. (40 × 7×)

DISCUSSION

Of the two moieties of the drug centrophenoxine, dimethylaminoethanol is an established neurotropic agent and p-chlorophenoxy acetic acid is a metabolic regulator influencing cellular respiration and glucidic metabolism in plant cells. Thuillier *et al.* (1959) could experimentally register the normalising effects of centrophenoxine on certain functions of the central nervous system on one hand and retardation of aging, yellowing and falling of leaves on the other. There are experimental evidences to the fact that centrophenoxine increases the glucose up-take, oxygen consumption and carbon dioxide production of the nervous tissue both *in vivo* and *in vitro* (Nickel *et al.*, 1963; Herrmann and Dittmann, 1971; Oeriu *et al.*, 1973).

The most general sign of cellular aging may be the slowing down of protein synthesis in the old as compared to the young cells (Kanungo *et al.*, 1970). A heavy loss of Nissl patches followed by a mild staining of the cytoplasmic RNA and protein, lends support to the fact that protein synthesis is blocked to a considerable extent with aging, and the cellular demand for proteins and specific amino acids is met by the cell by consumption of its cytoplasm. This results in chromatolytic changes. Zs.-Nagy and his group (1979) in their studies with monovalent electrolyte contents of the cell have experimentally supported the above fact. Thus, it appears that a drug which is capable of normalizing senile cells must have some direct or indirect impact on the protein metabolism of the cell. More recently, Zs.-Nagy *et al.* (1981) described that controphenoxine effectively protects the cellular membrane from free-radical attacks and also decreases the K-ion concentration of the cortical cells of old rats and consequently improves the ion permeability of the neurons and finally enhances the protein-synthesizing system of the cells. The studies of Zs.-Nagy's group were confined to ionic concentration of the cells with age and after centrophenoxine treatment.

As noted in the present experimentation, the intensity of synthetic activities of the nucleolus was directly proportional to the degree of cytoplasmic chromatolysis. In general, the nucleolus increases in size immediately before giving out buds, droplets and extrusion products to the cytoplasm. This is in accordance with the observations of LaVelle and LaVelle (1975) consequent upon axotomy of hamster facial neurons, Sharma and Manocha (1978) in the protein-deprived spinal cord neurons of squirrel monkey and Manocha and Sharma (1977, 1978) in the neurons of central motor cortex, cerebellum and dorsal root ganglion of experimentally malnourished squirrel monkeys. That the rate of synthesis of the nucleolar RNA corresponds to the rate of synthesis of total cell RNA of ribosomal type, has been experimentally shown. A relationship between the nucleolar nucleic acid content, rate of chromatolysis and the rate of ribosomal RNA synthesis has also been indirectly shown by several studies (Watson, 1968a,b; Smetana *et al.*, 1968; Price and Porter, 1972). Nilsson and Leick (1970) described that the *Tetrahymena* nucleolar system responds to the demands for more cytoplasmic ribosomes by breaking up the nucleolar material into small aggregates arranged around the periphery of the nucleus inside the nuclear envelope. Brown and Dawid (1968) suggested that the development of multiple nucleoli from the parent nucleuolus is accompanied by a 5000-fold amplification the ribosomal RNA genes and is concomitant with a vigorous synthesis of ribosomes. Zs.-Nagy *et al.* (1981) believed that the increased intra-cellular K-ion concentration results in a more condensed state of chromatin, involving a slowing down of the RNA-synthesis. They also showed that the drug centrophenoxine reduces the K-ion concentration of the cell by increasing the permeability of the cell membrane and thus

speculated that the drug possibly exerts its positive effects by correcting and enhancing the synthesis of essential proteins of the neurons.

In our studies we could note a striking increase in the Nissl substance and pyrnoninophility of the cell cytoplasm after drug-treatment. The active synthetic activity of the nucleolus was at the peak up to a 40 day-treatment of the cells. Only a few cells occasionally presented nucleolar budding process up to or after 60 days-treatment. The increased protein staining of the cytoplasm is a further support to the fact that the drug corrects and stimulates the protein-synthesizing machinery.

An increase in the activity of α-esterase, nestled in the cytoplasm and the endoplasmic reticulum, lends support to our observation that the drug restores the endoplasmic reticular system. While reviewing the literature on the lipofuscinolytic activity of the drug, Nandy (1979) speculated that the reduction of lipofuscin and the enzymatic changes caused by centrophenoxine afford evidence to the regenerative activity of the drug. He further suggested that the prominence of rough endoplasmic reticulum in the neurons on treatment with centrophenoxine is possibly an evidence of increased protein synthesis. The present investigation supports the speculation of Nandy (1979) and concludes that the drug exerts its positive effects on the aged neurons by way of regeneration of its protein-synthesizing machinery.

SUMMARY

Cytochemical investigations have been made to understand the possible effects of centrophenoxine on the general cytoplasm and the nuclei of senile Purkinje cells. An enhanced nucleolar activity was recorded in the senile and treated Purkinje cells of white rats. The nucleolus was observed to present hyper-activity, as evidenced by the process of budding and extrusion. The rate of budding, etc. was proportionate to the intensity of chromatolytic changes in the parikarya. The nucleolus was at the peak of its synthetic process by the 40th day of treatment. A frank regeneration of Nissl patches and increased levels of α-esterase, and decrease in the activity of acid phosphatase and simple esterase were also recorded in the Purkinje cells after 60-day drug-treatment. After 60 days' drug-treatment the nucleoli resumed a normal functioning. It is concluded that the drug exerts its effects in the senile neurons by regenerating the general cytoplasm and restoring the protein-synthesizing system which gets blocked with aging.

Acknowledgements: We are thankful to the Indian Council of Medical Research, New Delhi for the financial assistance. We are also thankful to M/s Promonta Laboratorie, Hamburg for their consistant help, encouragement and liberal supply of centrophenoxine.

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AGE-RELATED CHANGES OF ASCORBIC ACID CONTENT OF LIVER AND BRAIN OF COMMON INDIAN TOAD, *BUFO MELANOSTICTUS*

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(*Received* 6 *October* 1983)

Abstract—Ascorbic acid content reaches a peak level in liver in group III male toads and then declines significantly. But there is no significant age-related change in ascorbic acid in the brain of male toads or in liver and brain of female toads. Sex comparison indicates that in females the content declines in the liver of age-group III and increases in age-group II, and there is an increase in the brain of age-group III females over their male counterparts.

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INTRODUCTION

That ascorbic acid declines with age in kidney (Padhi, 1980), liver (Rao and Patnaik, 1973), skeletal muscle (Haseeb and Patnaik, 1973) of male garden lizards and in rats (Kanungo and Patnaik, 1964) is known. Its involvement in various metabolic functions has been discussed (Patnaik, 1971; Padhi, 1980). The present study aims at estimating the ascorbic acid content in liver and brain of the common Indian toad, *Bufo melanostictus*.

MATERIALS AND METHODS

Wild toads of different sizes of both sexes and of four different age-groups were collected.

Age-group	*Range of body wt. (G) male/female*	*Range of snout-vent length (cm) male/female*
I	4–14/6–15	3.4–6.0/4.0–6.2
II	17–30/16–34	5.8–7.7/6.0–7.7
III	35–44/36–47	7.5–8.5/7.3–9.1
IV	47–81/47–110	8.0–10.0/8.0–11.5

The animals were killed with a blow on the head, livers and brains were removed and transferred to an ice-cold petri dish with distilled water, cleaned, weighed, homogenized with 5% Trichloroacetic acid in a low speed all-glass homogenizer (Remi) and centrifuged in a Remi centrifuge at 2000 rpm for 10 minutes. The supernatant was taken for estimation following the method of Roe (1967) and modified by Tewary and Pandey (1964).

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RESULTS

Our results on male toads (Table 1) show that there is a significant increase of ascorbic acid content in liver in age-group III. It then declines, whereas there is no such significant change in brain. Females do not exhibit any age-related change of ascorbic acid content in liver or in brain.

Sex comparison on liver (Table 1) indicates that the content is high in group II and low in group III females where as in brain group III females show higher content than males.

DISCUSSION

That the ascorbic acid metabolism declines with age in mammals has been reviewed (Patnaik, 1971). In garden lizards, ascorbic acid declines with age in liver (Rao and Patnaik, 1973), skeletal muscle (Haseeb and Patnaik, 1973) and kidney (Padhi, 1980), but in brain it remained almost constant. Our results (Table 1) in toads show an almost similar trend. Such age-related changes were thought to be due to changes in the rate of synthesis in the kidney and in the rate of utilization in the respective tissues. Therefore, it may be suggested that a basically common pattern of aging changes occurs in these animals.

So far we are aware, there is no report on age related changes in relation to sex. Our results indicating a significant difference in ascorbic acid level in liver and brain might be due to its rate of utilization in those tissues.

TABLE 1. EFFECT OF AGE ON THE ASCORBIC ACID (AA) CONTENT OF LIVER AND BRAIN OF COMMON INDIAN TOAD, *BUFO MELANOSTICTUS* (VALUES ARE AVERAGE ± SEM, NUMBER IN PARANTHESES INDICATES ANIMALS USED. ('P' BETWEEN AGE GROUP I & II, II & III, III & IV & IV & I)

		Liver		*Brain*	
Sex	*Age group*	*Mg AA/100g. wet wt.*	*'p'*	*Mg AA/100g wet wt.*	*'p'*
M	I	11.78 ± 1.54 (7)	NS	30.77 ± 3.80 (12)	NS
A	II	9.63 ± 0.75 (12)	0.001	25.03 ± 2.23 (17)	NS
L	III	16.94 ± 0.76 (9)	0.02	20.83 ± 2.30 (9)	NS
E	IV	13.79 ± 0.90 (7)	NS	29.94 ± 3.36 (11)	NS
F	I	*14.50 ± 1.47 (8)	NS	*27.76 ± 2.42 (13)	NS
E	II	•12.34 ± 0.93 (12)	NS	*26.03 ± 2.55 (14)	NS
M	III	*12.69 ± 0.75 (7)	NS	•28.91 ± 3.34 (9)	NS
A	IV	*12.08 ± 0.95 (13)	NS	*24.68 ± 3.47 (10)	NS
L					
E					

Comparison of male and female toads with respective age-groups.
*Not Significant.
•Significance at 0.05 level.
*Significance at 0.01 level.

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EFFECT OF AGING ON THE STORAGE AND CATABOLISM OF VITAMIN A IN MICE

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Abstract—In A/J mice, the mean total storage of vitamin A in the liver increased from birth to a maximum at 216 days, and then decreased at 644 days. The rate of deposition of intraperitoneally injected 11,12-$^3H_2$-retinyl acetate in micellar form into stored retinyl ester in the liver was inversely related to the age of the mice, whereas rates of excretion of radioactive metabolites in the urine and feces were not systematically affected by age. The percent storage of a dose of radioactive vitamin A and the ratio of the amount stored to that excreted were higher in very young mice than in older mice. Vitamin A reserves in old mice (644 days), although lower than in adult mice (~200 days), were still high and fully adequate to meet nutritional needs.

INTRODUCTION

LIVER RESERVES of vitamin A in humans are very limited at birth, but then increase rapidly during the first few years of life to reach concentrations equivalent to, or sometimes higher than, the median adult value of approximately 100 μg of vitamin A, expressed as retinol, per g liver (Moore, 1957; Olson *et al.*, 1984). Thereafter the intake of vitamin A (USDHEW, 1974; USDA, 1980) and the median total liver reserves (Raica *et al.*, 1972; Mitchell, 1973; Suthutvoravoot and Olson, 1974; Olson, 1979b) do not change appreciably with age. Defects in fat absorption, abnormalities of liver and kidney function, or the ingestion of halogenated hydrocarbons, on the other hand, markedly reduce liver reserves of vitamin A (DeLuca, 1979; Olson, 1984). Surveys of vitamin A reserves in humans, however, are not sensitive enough to detect small changes in the metabolism and storage of vitamin A as a function of age. We consequently examined both the liver storage of vitamin A in healthy A/J mice from 9 to 644 days as well as determined the way that intraperitoneally injected radioactive vitamin A was stored in the liver, metabolized and excreted as a function of age.

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MATERIALS AND METHODS

Isotopes and chemicals

All-*trans* $^3H_2$-11,12 retinyl acetate was a generous gift from Hoffmann-LaRoche, Inc., Basel, Switzerland. All-*trans* retinyl acetate was obtained from the Sigma Chemical Co., St. Louis, MO and all-*trans* retinol from the Distillation Products Division, Eastman Kodak Co., Rochester, NY. All other chemicals and solvents were of reagent grade.

*Purification of $^3H_2$-11,12-all-*trans *retinyl acetate*

Ampoules of $^3H_2$-11,12-all-*trans* retinyl acetate in benzene that contained 0.2 or 1 mCi were stored under nitrogen at −17° in the dark. By use of 87% aqueous methanol as eluant at a flow rate of 2 ml/min, radioactive retinyl acetate from one ampoule was purified on a reverse phase μ Bondapak C_{18} column, which was linked to a Waters Model ALC/GPC 201 high-pressure liquid chromatograph and a Perkin Elmer variable-wavelength detector set at 328 nm. The retinyl acetate fractions, which eluted between 16 and 17 ml, were pooled, extracted with hexane, and taken nearly to dryness under nitrogen. By TLC analysis of an aliquot of purified radioactive retinyl acetate (R_f = 0.8–0.9) on silica gel in benzene:chloroform:methanol 4:1:1, the average purity of the purified radioactive all-*trans* retinyl acetate was 89%, with a range from 77 to 97%.

Preparation of a micellar solution of radioactive retinyl acetate for injection

The highly concentrated solution of purified radioactive retinyl acetate was immediately diluted with a small amount of acetone, Tween 40 (polyoxyethylene sorbitan monopalmitate), nonradioactive retinyl acetate, α-tocopheryl acetate, and 0.9% saline. The usual amounts of compounds present in 0.3 ml of the water-clear injection solution were 90 μg of retinyl acetate containing 6 μCi of tritium, 100 μg α-tocopheryl acetate and 15% Tween 40. The micelle size under these conditions is about 85,000 daltons (Olson and Herron, 1964).

Animals

Virgin male A/J mice and pregnant female A/J mice (Jackson Laboratories, Bar Harbor, ME) were fed commercial mouse/rat chow (Teklad Mills, Madison, WI), which contained approximately 24% protein, 4% fat and 6% fiber. The vitamin A content of the diet was 13.37 IU (4 μg retinol equivalents/g), of which 54% consisted of preformed vitamin A. Mice were housed in polystyrene cages (Maryland Plastics, Heraldsburg, MD) containing wood shavings in a temperature and humidity controlled animal room with a 12 hour light-dark cycle. Animals were weighed every 4 days.

Experimental procedure

Eighteen or 21 mice of a given age were housed 3 to a cage, except that a mother and her nursing litter were kept together. When excreta were collected, wood shavings were replaced by a raised wire-mesh screen with 1.5 or 3 divisions/cm for weaned or nursing mice, respectively. Each growing and adult mouse was injected intraperitoneally with 75–115 μg $^3H_2$-11,12-all-*trans* retinyl acetate (3–7.5 μCi) in a micellar solution, whereas nursing mice each received 15–22 μg labeled retinyl acetate containing 1.3–1.4 μCi (7 μg retinyl acetate/g body weight). Urine and feces were collected daily for eight days. Growing and adult mice, in groups of 3, were usually sacrificed at 2, 4, 8, 12, 16, 20 and 28 days, although some were sacrificed at times up to 45 days.

Collection and analyses of feces and urine

At daily intervals mice were transferred from the cage, and dry feces were collected in a vial. Any that were soaked with urine were first rinsed with 50% ethanol and blotted. Fecal samples were stored at −17° until analysis. Feces were ground and extracted twice with 5 ml methanol. Extracts were filtered under vacuum through a coarse, sintered-glass funnel containing a 1 cm layer of anhydrous Na_2SO_4. The volume of the filtrate was measured, and an aliquot counted for radioactivity. The efficiency of extraction of radioactivity was >90%.

Urine was transferred to a flask, and the screen and cage bottom were rinsed twice with 10 ml 50% ethanol and then once with 10 ml 95% ethanol. Urine samples were frozen and stored at −17° until analyzed for radioactivity.

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Extraction and analysis of liver

The liver was removed, blotted, weighed and stored at −17° until analysis. The whole liver was then mashed with anhydrous sodium sulfate and stored overnight in the dark at 4° under 6.0 ml chloroform (Olson, 1979b). Both spectrophotometric and colorimetric assays for vitamin A were conducted on the chloroform extract (Olson, 1979b), and an aliquot was counted for radioactivity. Retinyl esters were separated from retinol on an 8% deactivated alumina column eluted with an increasing proportion (0–20%) of acetone in hexane. Pooled fractions containing retinyl ester or free retinol were then assayed as indicated above (Olson, 1979b).

Measurement of radioactivity

Aliquots of the original injection solution, urine extracts, and fecal extracts were added to 10 ml of Bray's scintillation fluid (Bray, 1960) and counted in a Beckman LS-100 liquid scintillation counter. Aliquots of the chloroform extract of liver and samples in hydrophobic solvents were counted in a toluene-based scintillation fluid (5 g 2,5-diphenyloxazole and 0.1 g 1,4-bis[2-(5-phenyloxazolyl)] benzene in 1 L toluene). Quenching corrections for yellow color and for chemical components in the samples were determined from experimentally determined standard curves with dinitrophenol and chloroform, respectively, as the quenching substances.

Kinetic and statistical analyses

Excretion data were plotted on a semilogarithmic scale, analyzed by a curve-peeling technique (Shipley and Clark, 1972), and expressed as first-order rate constants for a fast phase (k_A, 0–2 days) and a slow phase (k_B, 2–8 days). Although additional kinetic components might be defined, a two-component analysis adequately described the plots. Conventional regression analyses were conducted of lines derived from curve peeling. Values generally are expressed as means ± the standard deviation (SD). Student's "*t*" test was used for the estimation of probability.

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RESULTS

Effect of the experimental procedure on the growth and survival of animals

Control A/J mice in our animal quarters grew at a rate in harmony with weight-for-age information provided by the Jackson Laboratories. After injection, growing mice either just maintained or lost some weight for 1–2 days, but then grew at a normal rate. Adult mice, after a small initial decrease in weight for 1–2 days, maintained normal body weights during the rest of the experiment. Suckling mice were more sensitive to the experimental procedure, in that only about one-third recovered from the trauma of injection and gained weight, whereas the others either just maintained or lost weight. Whereas mortality among growing and mature mice was low (~3%), mortality among nursing mice was around 40%. Only survivors were studied.

Dynamics of vitamin A storage in the liver as a function of age

Mean total reserves of vitamin A in the liver rapidly increased from 9 μg retinol in 17 day old mice to a maximum of 1159–1606 μg at around 200 days of age, and then fell significantly ($P < 0.001$) to 844 μg in 620 day-old mice (Table 1). The predominant form of vitamin A in the livers of mice of all ages was retinyl ester (93–100%). Administered radioactive vitamin A was rapidly stored in the livers of mice of all ages (Figure 1). Maximum storage of the administered radioactive dose occurred in ≤4 days in mice up to 70 days of age, in 10–18 days in 200-day-old mice and in >18 days in very old mice. Once stored in the liver, radioactive retinyl ester was metabolized slowly in mice of all ages.

TABLE 1. LIVER STORAGE OF VITAMIN A IN A/J MICE AS A FUNCTION OF AGE.

Group	Mean Age at Sacrifice	n	Body Weight	Liver Weight	Vitamin A*	
					Total μg	μg/g
1	644 days	17	29 ± 4 g	1.4 ± 0.3 g	844 ± 302	657 ± 271
2	216	18	32 ± 5	1.3 ± 0.2	1159 ± 187	929 ± 186
3	216	18	30 ± 3	1.2 ± 0.1	1606 ± 300	1350 ± 298
4	82	21	24 ± 3	1.1 ± 0.2	220 ± 34	209 ± 35
5	80	21	24 ± 3	1.1 ± 0.2	408 ± 92	378 ± 118
6	50	18	20 ± 3	1.0 ± 0.1	161 ± 44	168 ± 42
7	46	21	19 ± 3	1.0 ± 0.2	121 ± 30	119 ± 29
8	38	21	16 ± 4	0.8 ± 0.2	81 ± 18	98 ± 16
9	36	21	14 ± 5	0.8 ± 0.3	69 ± 17	96 ± 27
10	17	19	3.8 ± 1.9	0.16 ± 0.09	9 ± 5	64 ± 29

*Expressed as retinol.

The rate of excretion of radioactive metabolites of retinyl acetate in the urine and feces of mice of different ages

Typical results for the excretion of radioactivity in the urine and feces of mice during the 8 day collection period are given in Figure 2, and first-order rate constants for excretion are summarized in Table 2. Values of k_A and k_B for both urine and feces did not vary systematically as a function of age. Some smaller trends were nonetheless evident; for ex-

TABLE 2. FIRST-ORDER RATE CONSTANTS FOR THE DAILY EXCRETION OF RADIOACTIVE METABOLITES DURING 8 DAYS FOLLOWING THE INJECTION OF $^3H_2$-11,12-RETINYL ACETATE INTO NURSING, GROWING AND MATURE MICE.

Group	n*	Age†	Urinary Excretion			Fecal Excretion		
			k_A	k_B	r_B‡	k_A	k_B	r_B‡
1	18	620 days	1.8 day^{-1}	0.18 day^{-1}	−0.95	2.2 day^{-1}	0.15 day^{-1}	−0.86
2#	18	200	2.4	0.21	−0.84	1.7	0.079	−0.67
			1.9	0.086	−0.67	1.1	0.33	−0.88
3#	18	200	2.2	0.15	−0.92	1.7	0.20	−0.93
			4.3	0.25	−0.98	1.9	0.18	−0.94
4	18	69 ± 3	3.9	0.17	−0.91	2.3	0.17	−0.95
5	21	67 ± 3	1.1	0.10	−0.64	1.0	0.40	−0.93
6	21	38 ± 3	0.75	0.072	−0.52	3.6	0.17	−0.80
7	21	33 ± 3	2.1	0.11	−0.91	0.73	0.19	−0.70
8	21	25 ± 1	3.5	0.20	−0.98	2.4	0.11	−0.94
9	21	23 ± 1	4.3	0.22	−0.97	1.6	0.16	−0.80
10§	21	9 ± 2	1.5	0.21	−0.90	2.0	0.34	−0.94
Mean ± SD			2.5 ± 1.2	0.16 ± 0.06	–	1.9 ± 0.8	0.21 ± 0.10	–

*Number of animals, grouped 3 per cage, in the experiment.
†Age after the eight day experimental period.
‡Correlation coefficients for the regression analysis of phase B.
#Data were obtained from two different sets of animals within this age group.
§Nursing mice.

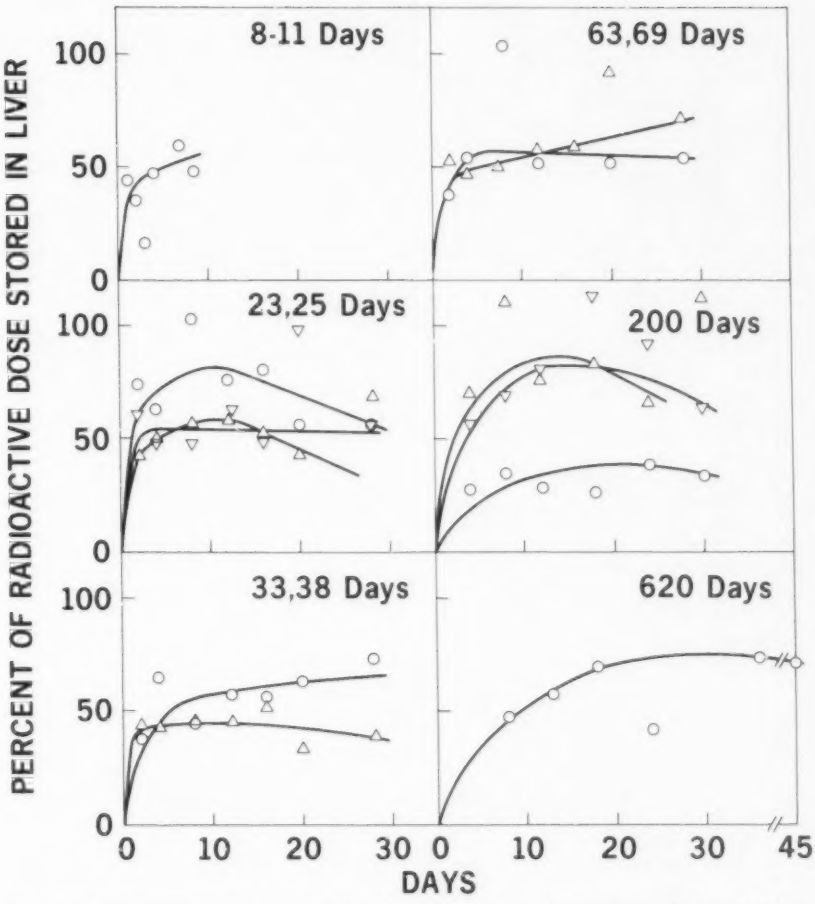

FIG. 1. The uptake and storage of injected radioactive retinyl acetate in the livers of mice of different ages.

ample, k_A and k_B for urinary excretion decreased from 21 to 38 days, followed by an increase to 200 days. Like changes were not noted, however, in fecal excretion. Because of the inherent variability in the data from such experiments, some age-dependent variation in excretion rates may therefore have been masked. The overall mean rate constants for the excreta were comparable, for example mean k_A values of 2.5 and 1.9 and mean k_B values of 0.16 and 0.21 for urine and feces, respectively.

Recovery and distribution of the administered dose of radioactive retinyl acetate

The distribution of radioactivity in the liver and excreta 8 days after its administration to mice of different ages is given in Table 3. No overall trend with age was noted, except that very young mice (groups 8–10) seemed to store more and to excrete less of the dose than did older mice. The mean total recovery of radioactivity from the liver and excreta in mice of all ages was 76 ± 12% of the administered dose.

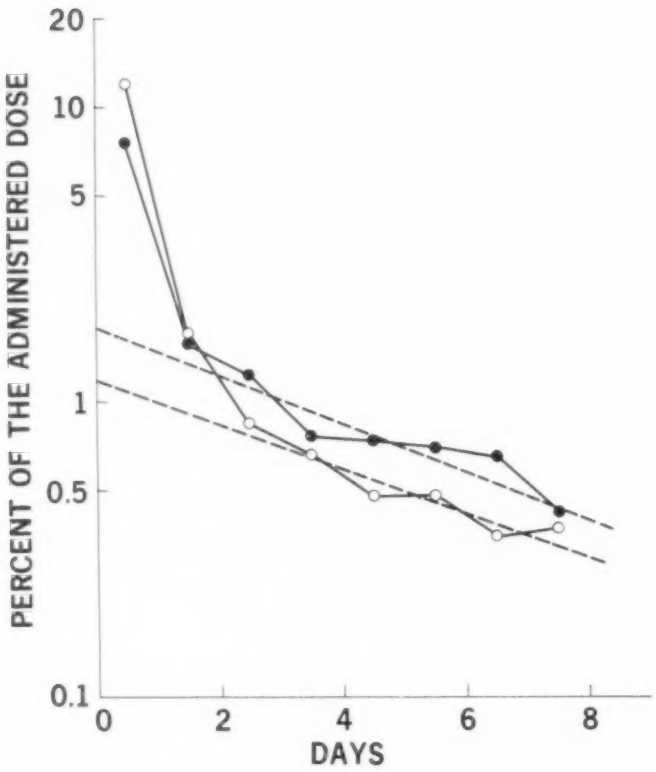

FIG. 2. Percent of the administered dose excreted daily in the feces (○) and urine (●) by 10-week-old mice (Group 4). The value for each 24 hour collection period is plotted as a midperiod point. The dotted regression lines are for the last 6 points.

DISCUSSION

The following relationships were noted in the present study: 1) the total amount and concentration of vitamin A in the liver rose from low levels in the newborn mouse to a maximum at approximately 200 days of age and then was lower in old (644 day) mice, 2) the rate of storage of a radioactive dose of vitamin A in the liver was more rapid in young mice than in old mice, 3) the ratio of the amount of vitamin A stored to that metabolized and excreted was generally greater in very young mice than in older mice, 4) the rate of excretion of metabolites of a radioactive dose of vitamin A in the urine and feces was not systematically affected by age, although some changes may have occurred during specific age periods, and 5) the turnover of vitamin A deposited in the liver was relatively slow in mice of all ages.

These observations can be rationalized in the following way. Five quantitative or semi-quantitative factors are known: the immediate rate of excretion (k_A), the slower rate of excretion (k_B), the concentration (and total amount) of vitamin A in the liver (Table 1), an estimate of the rate of deposition of administered vitamin A in the liver (Figure 1), and an estimate of dietary intake. The amount excreted per day is a sum of each of the given rate

TABLE 3. THE PERCENTAGES OF THE ADMINISTERED DOSE FOUND IN THE EXCRETA AND LIVER DURING 8 DAYS AFTER THE INTRAPERITONEAL ADMINISTRATION OF RADIOACTIVE RETINYL ACETATE.

Group	Age*	Urine	Feces	Liver	Total	Stored	
						Excreted	Percent stored of that recovered
1	620 days	8.9%	12%	48%	69%	2.3	70%
2	200	12	23	35	70	1.0	50
3	200	4.0	7.9	69	81	5.8	85
4	69	14	17	54†	85†	1.7	64
5	67	8.6	28	51	87	1.4	59
6	38	7.7	24	44	76	1.4	58
7	33	20	33	45	98	0.9	46
8	25	5.3	7.0	55	67	4.6	82
9‡	23	5.5	5.4	48	59	4.4	81
10#	9	8.7	8.2	48	65	2.8	74
Mean ± SD		9.5 ± 4.8	16.6 ± 9.9	49.7 ± 8.8	76 ± 12	2.6 ± 1.7	67 ± 14

*Age at the end of the 8-day experimental period.
†Storage and recovery at 4 days.
‡One of the 3 mice in the cage died midway in the period.
#Nursing mice

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constants times the appropriate pool. Although the age-independent rate constants k_A and k_B do not have specific physiological significance, the rate of catabolism of exogenously administered vitamin A contributes significantly to k_A, whereas the rate of metabolism of endogenous liver reserves will strongly influence k_B. In the latter regard, isotopic equilibrium between an administered dose and endogenous reserves of vitamin A in rats takes approximately 7 days (Rietz *et al.*, 1973).

In very young animals, the rate of deposition of an administered dose in the liver is rapid. Consequently the percentage of the administered dose remaining in the circulation will quickly decline. The product of k_A and that transient, diminishing pool will therefore be small. Because the liver reserves in very young animals are low, the product of k_B and that small liver reserve will also be small. In view of a slowly increasing dietary intake with age, up to maturity, liver reserves will gradually rise.

At 216 days of age, the rate of deposition of vitamin A in the liver is slower and liver reserves are much higher. Consequently the total amount excreted per day will be much greater than in very young mice and presumably will balance the intake.

In old mice (644 days), the rate of deposition in the liver is even slower, whereas liver stores are still high. In view of the same or slightly lower weight (Table 1), food intake is presumably slightly lower. These changes will tend to increase the excretion of vitamin A relative to the dietary intake, and will lead to a reduction in vitamin A reserves.

The inference should not be made, however, that the lower reserves noted in 644-day old mice pose any nutritional problem. The mean concentration of vitamin A in the liver of these mice was 657 μg/g, over 30-fold the presumed minimally adequate reserve of 20 μg/g (Suthutvoravoot and Olson, 1974), and the rate of turnover clearly was very slow (Figure 1). Once liver reserves of vitamin A are high, therefore, the depletion rate in adult or old mice, as in adult humans (Hume and Krebs, 1949; Sauberlich *et al.*, 1974), is very slow.

Only three past studies have examined the effect of age on vitamin A metabolism in rodents. In concordance with our observations, Carbini *et al.* (1976) found that the uptake of a radioactive dose of vitamin A by the liver reached a maximum earlier in 3-week old rats than in 8-week old rats, and that the rate of turnover of liver vitamin A was the same in both groups. In examining the rate of absorption of vitamin A from the small intestine, Hollander and Morgan (1979) found a 50% increase in the rate of vitamin A absorption in rats between 1.5 and 39 months of age, a change attributed to a decreased thickness of the unstirred water layer in the intestine of the older rats. In contrast, Fleming and Barrows (1982a) found no significant difference in the intestinal absorption of vitamin A nor in its distribution in tissues 18 hours after a radioactive dose was given to rats 6–24 months old. They further point out that the intestinal absorption of vitamin A in the studies of Hollander and Morgan (1979), when expressed per 100 g body weight, was higher at 1.5 months but then did not vary from 13–39 months of age. Because radioactive vitamin A in a micellar solution was injected intraperitoneally in our studies, however, the rates that we observed are not affected by any possible age-dependent differences in intestinal absorption.

Although not specifically focused on the effects of age, studies on the rate of excretion of administered radiolabeled vitamin A in the urine and feces of rats of different ages have been conducted in the past (Roberts and DeLuca, 1969; Varma and Beaton, 1972; Yeung and Veen-Baigent, 1974; Carney *et al.*, 1976; Sundaresan, 1977). By analyzing the data in these papers by a two-phase, curve-peeling method, the ranges of calculated values of k_A (1.2–2.6 day^{-1}) and of k_B (0.10–0.35 day^{-1}) agreed very well, in all but one case (Sundaresan, 1977), with those mean values of k_A and k_B observed in the present study with mice (a k_A of 1.9 and 2.6 day^{-1} and a k_B of 0.16 and 0.21 day^{-1}).

The discreet changes observed in vitamin A storage as a function of age thus relate more closely to the rate of deposition of an administered dose of vitamin A in the liver rather than to its rate of catabolism and excretion. In the case of other vitamins, such as vitamin B_6 (Fonda *et al.*, 1980) vitamin B_{12}, and niacin (Fleming and Barrows, 1982b), the absorption, uptake by liver and other organs, and in the case of vitamin B_6, its phosphorylation, were not age-dependent. In those studies, however, mice 10–33 months of age (Fonda *et al.*, 1980) and rats 6–24 months of age (Fleming and Barrows, 1982) were employed. Thus, the age span examined in those investigations was different than in the present study (1 day–644 days). Nonetheless, age clearly may affect the overall metabolism of one essential nutrient in a manner quite different from that of another.

SUMMARY

In A/J mice (17–644 days old), the mean total storage of vitamin A in the liver increased from 9 μg, expressed as retinol, at 17 days to 1159–1606 μg at 216 days, and then decreased to 844 μg at 644 days. The rate of deposition of intraperitoneally injected 11,12-$^3H_2$-retinyl acetate in micellar form into stored retinyl ester in the liver was inversely related to the age of the mice. The first-order rate constants for the rapid (k_A) and slow (k_B) excretion of radioactive metabolites in the urine (mean values of 2.5 ± 1.2 day^{-1} and 0.16 ± 0.06 day^{-1}, respectively) and in the feces (mean values of 1.9 ± 0.8 day^{-1} and 0.21 ± 0.10 day^{-1}, respectively), however, were not systematically affected by age. The percent storage of a dose of radioactive vitamin A and the ratio of the amount stored to that excreted was higher in very young mice than in older mice. The change in liver storage as a function of age is rationalized in terms of the defined kinetic parameters. Vitamin A

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reserves in old mice (644 days), although lower than in adult mice (~ 200 days), were still high and fully adequate to meet nutritional needs.

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EFFECT OF MATING STATUS ON *DROSOPHILA VIRILIS* LIFESPAN

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Abstract—Mating status markedly affected the lifespan of *Drosophila virilis* flies but the effects of virginity on lifespan were different between the sexes. In all cases lengthening of female lifespan and shortening of male lifespan were observed as an effect of virginity. Lifespan was also affected either by the population density or by the sex ratio per culture. At a sex ratio (proportion of females) of 0.5, lifespan was constant in both sexes irrespective of the population density and females lived significantly longer than males. At high (0.9) and low (0.1) sex ratios mean lifespan did not differ between sexes, but it was longer at high sex ratios than at low ones. A negative correlation between mean lifespan and the number of males per vial was revealed for both sexes. Effects of mating on egg-laying activity were also analyzed. Although the number of eggs laid during the first 20 days period after eclosion was greater in mated females than in unmated virgin females, lifetime egg production was not always larger in the former than in the latter. No particular relationship between egg-laying activity and female lifespan was proven. The lifespan of *D. virilis* flies seemed to be determined by complex interactions between both sexes, in which the sexual activity of males may play the most important role.

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INTRODUCTION

A SEXUAL DIFFERENCE in lifespans is a well known phenomenon in various animals. However, in insects, on which many detailed analytical studies on lifespan have been conducted, it is still difficult to conclude which sex can live longer, because adult lifespan depends on both the genetic composition of an organism and various environmental factors.

Mating status has been considered to be one of the important environmental factors affecting the lifespan of adult flies (Lamb, 1978). Although there have been some previous studies on the effects of mating status on adult lifespan, their results were not consistent. Several groups have observed that *Drosophila* virgin females live longer than mated females (Maynard Smith, 1958; Malick and Kidwell, 1966; David and Cohet, 1971; Giess, 1977; Ohba, unpublished).

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Maynard Smith (1958) reported that *Drosophila subobscula* heat-treated sterile females could survive as long as virgin females and concluded that the intense activity of egg-laying by mated females caused the shortening of their lifespan. Lamb (1964) and Nöthel (1965) also supported this view from their experiments in which X-ray irradiation of low doses had induced both partial sterilization and prolongation of female lifespan. These results are, however, indirect evidence that the acceleration of activity as a result of mating actually caused the shortening of female lifespan.

There is also some negative data which does not support that explanation (Kidwell and Malick, 1967; Sonnenblick and Grodis, 1963). On the whole, it can be said that "quite clearly there is a complicated interrelationship between fecundity, environmental conditions and the longevity of female flies" (Lamb, 1978).

Concerning the effects of mating status on the lifespan of male flies, comparatively little is known at present. Bilewicz (1953) reported that the lifespan of mated *Drosophila melanogaster* males was slightly shorter than that of the virgin males. On the contrary, Ohba (unpublished) recently found that virgin *Drosophila virilis* males have a shorter lifespan than mated males, while mating has shortened the life span in females. With the housefly (*Musca domestica*), Ragland and Sohal (1973) found that individually isolated virgin males lived longer (mean lifespan = about 50 days) than those reared in mass (mean lifespan = 20–30 days), and that mated males had the shortest mean lifespan (14–15 days). The sex ratio in a culture also affected the lifespan of both sexes. Similar results have been found in a recent experiment (Buchan and Sohal, 1981) with the explanation being that changes in physical activity due to mating behavior affected the lifespan.

The problem of the effects of mating status on lifespan is further complicated by the fact that population density affects the lifespan of both sexes (Pearl, *et al.*, 1927; Rockstein and Miquel, 1973; Lamb, 1978). In usual experiments the population density changes as a result of the changing mating status or sex ratio. In studying the problems of mating status therefore, care must be taken to consider the interrelationship between sex ratio and population density.

Undoubtedly the variation in lifespan caused by mating status seems to be connected with sex specific characters, but the underlying mechanisms are still unsolved. The aim of the present study is to reconfirm the relationship between mating status and lifespan for both sexes and to obtain further information about the mechanisms underlying these relationships. For this purpose, we examined in detail the lifespan of *Drosophila virilis* at various sex ratios and population densities. The effect of mating on both egg-laying activity and lifespan was also investigated to determine the correlation between reproductive activity and lifespan in different mating status.

MATERIALS AND METHODS

Strains

In this study *Drosophila virilis* was used because extensive studies on the longevity and aging of this species are being conducted in this laboratory. Most of the present experiments were carried out on the inbred strain Tokyo (abbr. TK) which has been maintained in this laboratory for more than thirty years in small mass culture. In addition, 10 isofemale strains isolated from a natural population near Matsuyama in 1973 (Ohba and Sone, in preparation) were also used.

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Culture media

Insect cultures were raised on the standard aceptic yeast medium II which contained the following in 100 ml of distilled water: dried brewer's yeast "Ebios" (8 g); agar powder (1 g); cane sugar (5 g); propionic acid (0.5 ml); and paraoxyhydroxybenzoate "Bokinin" (0.1 g in 1 ml of 70% alcohol solution).

For measuring adult lifespan, flies were fed on standard medium III in which the sugar content was doubled over that of standard medium II described above.

Flies

Flies raised on standard medium II at 25°C were collected and sexed without etherization within 24 hours after eclosion. In this species copulation never occurred during this period. According to the experimental design stated below, a given number of flies were put in a vial (30 mm in diameter, 105 mm in height) with about 5 ml of culture medium and a cotten plug. Flies were placed in fresh vials every two days and dead flies were counted and removed at the time of transfer. All culture vials were kept in a constant temperature room at 25 ± 0.5°C with permanent lighting (about 200 lux).

Mating status

Three kinds of experiments were performed.

1) The lifespan of virgin TK flies kept at a density of 20 females or males per vial was compared with the lifespan of freely mated TKs kept at a density of 10 females and 10 males per vial. A total of 180 flies were examined for each group. 10 isofemale lines were also examined for their lifespan in the same way as the TKs but the number of flies examined varied from 40 to 150 for each sex of each strain.

2) Under various conditions of different sex ratios and population densities, the lifespan of the TK flies was studied in the second experiment. The total number of flies per vial ranged from 2 to 20 and the sex ratio, expressed as the proportion of females per total, was changed from 1 to 0.

3) The effects of mating on egg-laying and lifespan for females were studied in all combinations of two female strains (TK and A12) and four male strains (TK, A12, A35 and B31). In this experiment the lifespan was compared between virgin flies kept individually in a vial and mated flies in which a single female was mated to three males. The number of replications was five for each condition. Throughout the whole period of female lifespan, the number of eggs laid on the medium in a two day interval was counted after the flies were transferred to new vials. Although analysis of survival curves for the flies is desirable, it was impossible because the total number of flies was only 5 females and 15 males in each condition.

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RESULTS

Survival curves of the TK strain

Figure 1 shows the survival curves of four groups of the TK flies; virgin and mated females, and virgin and mated males. Clearly, virgin females lived significantly longer than mated females. The mean lifespan was 87.64 days for the former and 72.57 days for the latter (Table 1). However, virgin males lived shorter than mated males, with the mean lifespan of 50.10 days (virgin) and 60.19 days (mated). These results agreed with those of a previous experiment in which a slightly modified medium was used and the mean lifespan in days was 97.05 (virgin females), 82.35 (mated females), 52.64 (mated males) and 40.37 (virgin males), respectively (Ohba, unpublished). From these results it can be concluded that the mean lifespan of the inbred TK strain differs between the sexes and that mating status has a reverse effect on survivorship of female and male flies.

Effects of mating on lifespan of other strains

To ascertain whether mating effects on lifespan, as observed for the TK strain are a common phenomena, the lifespan of 10 isofemale strains from the Matsuyama population was examined under the same experimental conditions as the TK strain. The results are summarized in Table 1 together with the data on TK noted above. In 9 out of 11

TABLE 1. MEAN ADULT LIFESPAN (± S.E.) OF 11 DIFFERENT STRAINS OF *D. virilis*, UNDER THE EXPERIMENTAL CONDITION OF STANDARD MEDIUM 111, 25°C, 20 FLIES PER VIAL.

	Female				*Male*			
	Virgin		*Mated*		*Mated*		*Virgin*	
Strain	*Mean ± S.E.*	*N**	*Mean ± S.E.*	*N**	*Mean ± S.E.*	*N**	*Mean ± S.E.*	*N**
A 11	76.65 ± 2.57	(40)	63.12 ± 1.69	(150)	71.11 ± 2.05	(150)	46.00 ± 1.65	(80)
A 12	76.45 ± 2.63	(40)	73.69 ± 1.46	(150)	80.04 ± 1.62	(150)	60.05 ± 2.93	(40)
A 13	84.82 ± 1.43	(100)	87.59 ± 1.57	(150)	90.63 ± 2.09	(150)	71.66 ± 2.56	(100)
A 35	100.70 ± 1.48	(80)	101.97 ± 2.10	(150)	80.79 ± 2.38	(150)	72.93 ± 3.10	(80)
B 12	86.05 ± 1.85	(80)	73.68 ± 1.65	(150)	60.52 ± 1.70	(150)	51.23 ± 1.60	(120)
B 15	84.50 ± 2.51	(80)	75.89 ± 1.69	(150)	83.48 ± 2.10	(150)	66.23 ± 1.95	(80)
B 22	84.10 ± 1.56	(100)	69.95 ± 1.61	(150)	91.97 ± 1.84	(150)	110.86 ± 1.74	(100)
B 31	80.85 ± 3.26	(40)	63.16 ± 1.15	(150)	71.59 ± 2.21	(150)	67.00 ± 2.57	(40)
B 33	72.86 ± 1.27	(100)	60.88 ± 1.69	(150)	63.63 ± 1.11	(150)	63.32 ± 1.39	(100)
B 42	94.78 ± 2.08	(80)	75.89 ± 1.78	(150)	84.84 ± 2.16	(150)	78.20 ± 2.61	(80)
TK	87.64 ± 1.10	(180)	72.57 ± 1.10	(180)	60.19 ± 1.02	(180)	50.10 ± 1.08	(180)
Average	84.49	(11)**	74.40	(11)	76.25	(11)	67.05	(11)

*Number of flies examined
**Number of strains

strains including TK, virgin females lived longer than mated females. In the remaining two cases the difference was statistically not significant. Averaging the 11 strains, the lengthening effect of virginity on female lifespan was 13.6%. With male flies, mating status has a marked effect on the mean lifespan. Unmated virgin males always lived

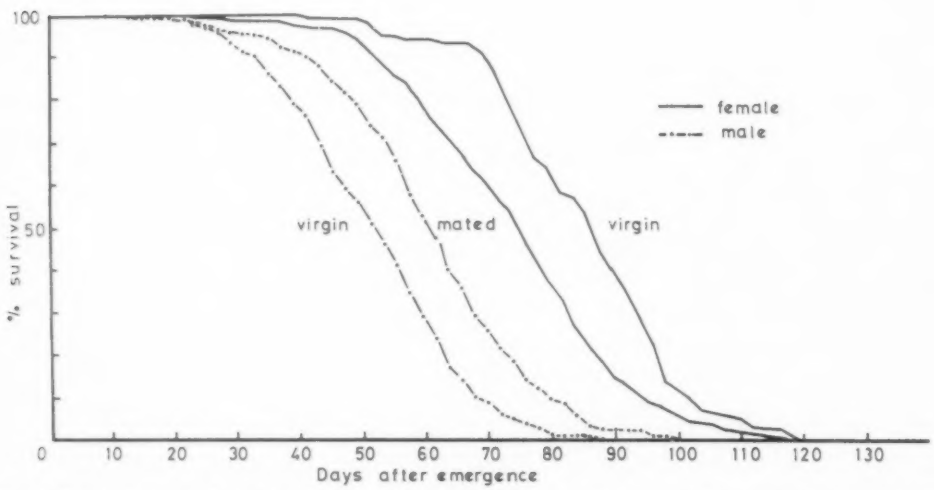

FIG. 1. Survival curves of mated and unmated flies of the TK strain. Culture conditions: standard medium III, 25°C and 20 flies per vial.

shorter than mated males, with the one exception of the B22 strain. The shortening effect of virginity on male lifespan was 12.1% on the average. Summarizing the results obtained in the present study, it can be said that the effect of virginity on adult lifespan differs between sexes in *D. virilis* with a lengthening of female lifespan and a shortening of male lifespan.

The data shown in Table 1 suggest significant interstrain differences in adult lifespan. This point will be studied in details in a future paper (Ohba and Sone, in preparation), together with the problem of sexual difference in lifespan.

Effects of sex ratio and population density on lifespan of the TK flies

Table 2 represents the mean lifespan of the TK flies under various combinations of sex ratio and population density. The mean lifespan varied from 47.4 to 81.8 days in males and from 57.4 to 98.3 days in females. In cases where equal numbers of females and males coexisted in a vial (Series I: sex ratio = 0.5), the mean lifespan was constant in both sexes (about 71 days for females and 57 days for males), irrespective of population density in the range from 4 to 20 flies per vial.

In the experiments of Series II, in which the ratio varied from 1 to 0 under a fixed population density (20 flies per vial), the sex ratio affected the lifespan of both sexes. As seen in Table 2 and Figure 2, a sexual difference was not apparent in two cases of extreme sex ratio, 0.9 and 0.1. At a high sex ratio (0.9), females and males had similar mean lifespans, 79.32 and 81.80 days respectively. At a low sex ratio (0.1) too, the difference be-

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TABLE 2. EFFECTS OF SEX RATIO AND POPULATION DENSITY ON THE MEAN LIFESPAN OF THE TK STRAIN OF *D. virilis* UNDER CULTURE CONDITIONS OF STANDARD MEDIUM III AND 25°C.

Series	No. of flies per vial	Sex ratio*	N**	Lifespan	
				Female Mean ± S.E.	Male Mean ± S.E.
I	20 (10♀ + 10♂)	0.5	80	70.05 ± 2.01	57.70 ± 1.90
	10 (5♀ + 5♂)	0.5	80	70.65 ± 2.46	55.10 ± 1.47
	4 (2♀ + 2♂)	0.5	80	71.50 ± 2.99	57.85 ± 1.98
II	20 (20♀ + 0♂)	1.0	100	81.18 ± 1.38	–
	20 (18♀ + 2♂)	0.9	200	79.32 ± 1.09	81.80 ± 3.04
	20 (10♀ + 10♂)	0.5	100	73.24 ± 1.84	60.08 ± 1.53
	20 (2♀ + 18♂)	0.1	200	57.40 ± 2.96	52.85 ± 1.18
	20 (0♀ + 20♂)	0.0	100		53.16 ± 1.38
III	20 (20♀ + 0♂)	1.0	40	95.00 ± 1.62	–
	10 (10♀ + 0♂)	1.0	40	91.15 ± 1.97	–
	5 (5♀ + 0♂)	1.0	40	90.40 ± 1.82	–
	2 (2♀ + 0♂)	1.0	40	98.25 ± 1.81	–
	20 (0♀ + 20♂)	0	40	–	47.40 ± 2.12
	10 (0♀ + 10♂)	0	40	–	57.95 ± 2.77
	5 (0♀ + 5♂)	0	40	–	62.60 ± 2.36
	2 (0♀ + 2♂)	0	40	–	67.60 ± 2.37

*Proportion of female files.
**Total number of flies examined.

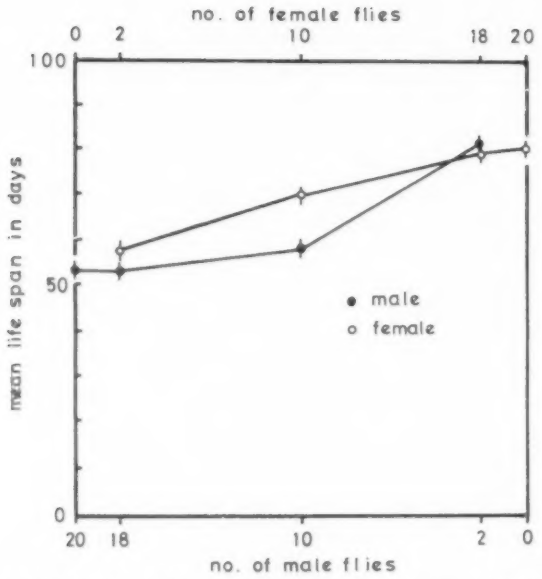

FIG. 2. Effects of sex ratio (proportion of females in a vial) on mean lifespan of the TK strain of *D. virilis* at a fixed population density of 20 flies per vial. Bars represent standard error.

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tween the mean female (57.40 days) and male (52.85 days) lifespans was statistically insignificant. These values were very close to the mean lifespan of unmated virgin females (81.18 days) and males (53.16 days).

In the experiments of Series IiI in which flies were kept unmated (sex ratio = 1 or 0), population density effects were found only in males. As the number of male flies per vial increased from 2 to 20, the mean lifespan decreased gradually from 67.60 days to 47.40 days. Contrarily, no clinal change associated with density was noticed in female virgin flies.

From the data shown in Table 2, the correlation coefficients between mean lifespan and sex ratio and population density factors were calculated for both sexes. As shown in Table 3, there was a highly significant positive correlation between female lifespan and sex ratio ($p < 0.001$). A negative correlation between lifespan and number of males was also significant for both sexes ($p < 0.001$ for females and $p < 0.01$ for males).

Effects of mating status on egg-laying activity and lifespan

To analyze the causal relationship between reproductive activity and lifespan for female flies, it is indispensable to examine egg-laying activity and survivorship for each female. In these study lifetime egg-laying activity was compared between mated and unmated virgin females of two different strains, TK and A12. Because the number of females was only five for each experimental condition, larger fluctuations in the data were inevitable. But preliminary analysis of variance revealed no significant differences among four strains of males used in this experiment. Therefore, data on females mated to four different male strains were pooled as mated females.

TABLE 3. CORRELATION COEFFICIENTS BETWEEN MEAN LIFE SPAN AND SEX RATIO AND POPULATION DENSITY FACTORS.

	Lifespan	
	Male	*Female*
No. of females/vial	0.6558*	0.2790
No. of males/vial	−0.7099**	−0.8421***
No. of total flies/vial	−0.1347	−0.4136
Sex ratio	0.5619	0.9307***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

In Table 4 the mean number of eggs laid by a female is shown for two different periods, the first 20 days after eclosion and for the whole lifetime. Under the present conditions (1 female + 3 males per vial, standard yeast medium III, 25°C, renewal of medium at 2 day intervals), mean lifetime egg production always exceeded one thousand in both strains. According to our past experience, unmated virgin females of the TK strain can lay as many eggs as mated females. This fact was proven in the present experiment. Lifetime mean egg production was 1076 for a virgin female and 1042 for a mated female. For the A12 strain, however, lifetime egg production of a mated female (1416) was about 1.4-fold that of a virgin female (1001). On the other hand, for the period of the first 20 days after eclosion there was a highly significant difference between mated and unmated females in both strains ($p < 0.001$). This fact suggests that the pattern of egg-laying may differ between virgin and mated females. This difference is clearly seen in Figure 3. In both the TK and A12 strains, mated females had a sharp peak of egg-laying at an earlier stage of adult life, just after 10 days from eclosion. In contrast, egg production in virgin females gradually increased until about 30 days after eclosion. In the TK strain the period of egg-laying in virgin females was longer than that in mated females. The difference was more than 20 days (Figure 3). However, this was not the case with the A12 strain in which both virgin and mated females had a similar egg-laying period, about 70 days (Figure 3).

TABLE 4. EFFECT OF MALE PRESENCE ON EGG-LAYING OF *D. virilis* FEMALES.

		No. of eggs per female			
Strain	*Periods*	*Virgin*	*Mated*	*t*	*P*
TK	First 20 days	400.0 ± 40.8	661.7 ± 28.4	4.480	<0.001
	Life time	1076.2 ± 71.7	1041.5 ± 68.3	0.254	n.s.
A 12	First 20 days	256.2 ± 63.9	688.1 ± 30.0	6.819	<0.001
	Life time	1001.2 ± 215.8	1415.7 ± 107.9	1.853	<0.05

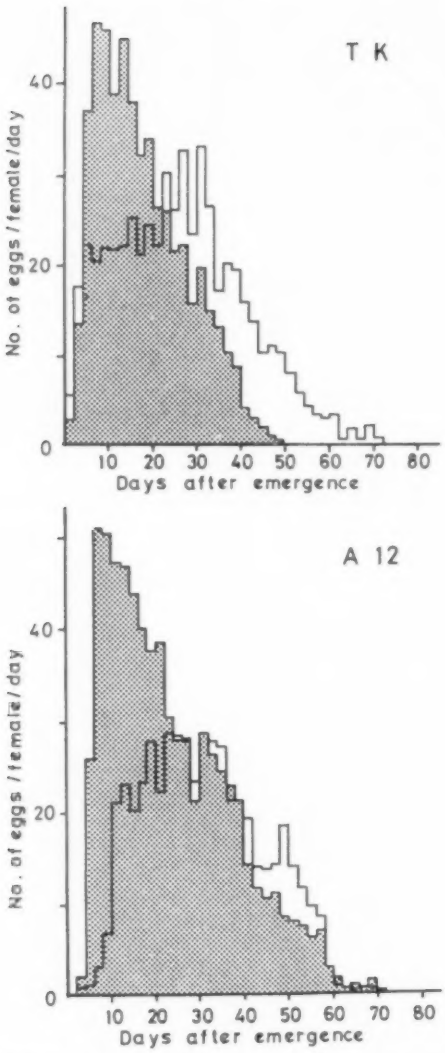

FIG. 3. Age-associated changes in the egg-laying activity in virgin and mated females in TK and A12 strain. Shaded (1 female with 3 males per vial) and open (1 virgin female per vial) bars indicate the mean number of eggs per female per day.

For all flies on which egg-laying activity was surveyed, the mean life span was calculated (Table 5). Because the number of females was only 5 for each condition, fluctuation of the data was inevitable. But the following can be said from the data. When TK virgin females were kept individually, their mean lifespan was 106.0 days, being about 1.5 times longer than that of mated females of the same strain, 71.56 days. This result parallels those shown in Figure 1 and Table 1, in which 20 flies were kept in vial. But this lengthening effect of virginity on female life span was not apparent for the A12 strain, where the difference between mean lifespans for virgin and mated females (74.40 and

TABLE 5. COMPARISON OF MEAN LIFESPAN (± S.E.) BETWEEN MATED (1 + 3) AND UNMATED VIRGIN (1) FEMALES OF *D. virilis*, UNDER CULTURE CONDITIONS OF STANDARD MEDIUM III AND 25°C.

Strain		*Mated*				
Female	*Male*		*Overall*	*Virgin*	*t*	*p*
A12	A12	65.00 ± 6.14 (4)*	69.89 ± 3.32 (18)	74.40 ± 4.83 (5)	0.606	n.s.
	B31	73.60 ± 5.88 (5)				
	TK	73.60 ± 6.70 (5)				
	A35	65.50 ± 9.22 (4)				
TK	A12	58.00 ± 6.38 (4)	71.56 ± 5.72 (18)	106.00 ± 8.63 (5)	2.915	$p < 0.005$
	B31	76.50 ± 14.78 (4)				
	TK	76.80 ± 12.13 (5)				
	A35	73.20 ± 12.18 (5)				

*Number in parenthesis indicates the number of females tested.

69.89 days, respectively) was statistically insignificant. It should be noted that in the previous experiment shown in Table 1, no significant difference in mean lifespan was found between virgin and mated females of A12 strain.

For both the TK and A12 strains, it is shown that female mean lifespan did not change with different male strains with which the females coexisted (Table 5). Presumably, the effects of mating on female lifespan depend on the genetic architecture of the female strain, not on the male strain.

DISCUSSION

Sex difference in lifespan is a rather common phenomenon and many authors have referred to this problem in *Drosophila*. In this connection it has also been shown that lifespans depend on the mating status of flies. In many cases virgin females could live longer than mated females. Egg-laying activity was proposed by some workers to be responsible for this difference. Maynard Smith (1958) concluded that the shorter lifespan of mated *D. subobscura* females, when compared to virgin females, was probably due to the increased number of eggs laid by mated females. Lamb (1964) also supported this correlation between egg-laying activity and female lifespan from her experiments on irradiated *D. subobscura* females.

In the present study on *D. virilis*, however, we could find no evidence for a correlation between female lifespan and egg-laying activity. In this species, virgin females live longer than mated females, though the difference varies among strains. Averaging the 11 tested inbred strains, the mean lifespan was about 84.5 days for virgin females and 74.4 days for mated females, respectively. As to the lifetime number of eggs laid per female, no difference was found between virgin and mated females of the TK strain, in which a marked and constant difference in lifespans was revealed between virgin (87.6 days) and mated (72.6 days) females. In the A12 strain in which the difference between virgin and mated females is very small or insignificant (76.6 and 73.7 days, respectively), lifetime egg production of a mated female is about 1.4 times greater than that of a virgin female. It can be concluded therefore, with the majority of the tested strains, that virginity usually has a

marked lengthening effect on female lifespan. At the same time, virginity had a variable effect on total egg production in two tested strains. In the course of our serial studies on *D. virilis* lifespan, we often found results which indicate no correlation between the number of eggs laid by a female and her lifespan. Further data will be presented elsewhere (Aigaki and Ohba, 1984; Ohba *et al.*, in preparation).

In regard to the effect of mating status on the lifespan of male flies, only a few studies have been reported. In the housefly (*Musca domestica*) the lifespan of males reared with males is longer than those reared with females (Ragland and Sohal, 1973). The elongation of mean lifespan is most conspicuous when males are kept individually in a vial. For *Drosophila* species, no paper has appeared reporting a shorter lifespan in virgin unmated males than in mated ones. However, this is the case of the present study. In 10 out of 11 inbred strains examined, virgin unmated males had a shorter mean lifespan than mated males. Averaging the 11 strains, the mean lifespan of the former was 67.1 days while that of the latter was 76.3 days. A shortened lifespan in virgin males has been repeatedly observed in *D. virilis* on different culture media and in *D. melanogaster* (Ohba, unpublished). This may be a general phenomenon at least in *Drosophila* species.

In regard to the effects of population density on lifespan, Pearl and coworkers (Pearl *et al.*, 1927; Pearl, 1928) reported the density dependent lifespan reduction in *D. melanogaster*. Under their experimental conditions optimal densities for adult lifespan were found between 35 and 55 flies per one ounce bottle. Both above and below this range the mean lifespan was gradually reduced. Because both sexes were treated together, separate information on females and on males was not reported.

Recently Rockstein *et al.* (1981) concluded that the median and maximal lifespans of adult houseflies were increased as the number of flies per cage was decreased from 240 to 10. This tendency was common to both sexes. Their results were explained by an increase in physical activity with density, including interaction among individuals in a restricted cage.

In our experiments in which males or females were kept separately in a vial, density-dependent lifespan changes were found in males but not in females. The mean lifespan decreased linearly from 67.6 days at a density of 2 males per vial to 47.4 days at 20 males per vial. No significant changes occurred in the range from 2 to 20 females per vial. In cases where equal numbers of males and females were put together in a vial, no change in mean lifespan was observed for both sexes in the range of 4–20 flies per vial. These results suggest a complicated interrelationship between population density and sex ratio.

The results in the present study clearly indicate that sex ratio affects the mean lifespan of both sexes. At a fixed population density of 20 flies per vial, there was no difference in female mean lifespan between a sex ratio (proportion of females) of 1.0 and 0.9. In the reverse cases in which the ratio was 0.1 and 0, the mean lifespan of males did not differ significantly. In the sex ratio range of 0.9 to 0.1, however, mean lifespans increased in both sexes as the sex ratio increased.

Statistical analysis on the present data shows a negative correlation between the number of males in a vial and the lifespan of both sexes and a positive correlation between the sex ratio and female lifespan. These facts indicate that the factor(s) responsible for lifespan shortening are confined mainly to males. Without the presence of males the factor(s) is ineffective. Its effects are enhanced with male density and repressed with female density. It was also confirmed that the factor(s) was different from those affecting female reproductive activity (oviposition).

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Two kinds of explanation may be possible for the life shortening factor(s) in *Drosophila* males. One is that the presence of males causes a change for the worse in environmental conditions. With an increasing number of male flies, the culture media may become more toxic or infectious due to substances excreted by males. The fact that infectious agents reduce the lifespan of *D. melanogaster* (Van Herrewege and David, 1973) and that the interval of medium exchange affects the lifespan (Lamb, 1964) may suggest this possibility. Another explanation is that male physical activity, especially reproductive activity, is involved in life shortening. In the housefly (*Musca domestica*), increased physical activity markedly changes the lifespan of both sexes, especially in males (Ragland and Sohal, 1973; Rockstein *et al.*, 1981). In studies of physiological differences among flies raised at different temperatures, David and Cohet (1971) and Cohet and David (1973, 1974, 1976) reported that *D. melanogaster* males raised at 25°C had the most harmful effects on female lifespan and females raised at 25°C were the most resistant to copulation. They considered that more aggressive males had more harmful effects through their higher copulating activity. In male populations of *D. virilis* we often observe active aggressive behavior in compensation for mating behavior. In populations which include both sexes, when females are in a majority (high sex ratio), this kind of aggressive behavior is rare and, if it occurs, it is ended quickly after success in mating. In the cases of low sex ratios, males are more excited and aggressive because of the difficulty in mating. Such increased behavioral activity for males ultimately results in a deleterious effect on lifespans of females as well as males.

Between the above two explanations of lifespan shortening factor(s) in males, the latter is more probable and important for explaining the present data, although the possibility of the former can not be completely denied. In the former case the effects of males on lifespan are assumed to be one-sided, independent of the presence or the number of females. On the other hand, the effects are the results of interactions between males and females in the latter case. All our data indicates complex interrelationships between sex and density.

In a recent paper Partridge and Farquhr (1981) reported a significant difference in lifespan for *D. melanogaster* males kept with one virgin females and those with eight virgin females per day. A clear difference in lifespan was also found between males kept with virgin and inseminated females. The relationship between insemination rate (number of females inseminated by a male per 2 days) and age differed between males kept with one and eight females. From these results they concluded that the increasing sexual activity of male *D. melanogaster* reduced its longevity and that this was a significant physiological cost of male sexual activity. Their conclusion on the cost of male sexual activity was based on their data on the activity of a male per culture. However, male reproductive activity is affected by the number of males, as shown in our present data in which the number of males was changed and shortening of lifespan occurred when males outnumbered females. This lifespan shortening effect is assumed to be determined by the possibility for copulation of each individual male. In other word, lifespan may depend on both female receptivity and male activity in copulation. These characteristics are under the control of the genetic architecture of both sexes and are influenced by some environmental factors including sex ratio and population density.

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METABOLIC PROFILES OF RATS OF DIFFERENT AGES

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Abstract—Urine samples from 6- to 31-month-old male Fischer F344 rats were analyzed using a high performance liquid chromatograph and a unique computer-based data analysis and quality control system in order to discover substances that could be used as markers in the aging process. Metabolic profiles of the organic acids from these urines yielded 42 peaks whose areas could be measured reliably. Of the 42 peaks, 10 were found by analysis of variance to vary significantly ($p < 0.05$) with age. Rats from the four ages could also be distinguished using a multivariate statistic (discriminant analysis).

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INTRODUCTION

AN IMPORTANT goal of much of the current research on aging is to develop a set of biomarkers that can be used to study the underlying biochemical mechanisms of aging or to monitor the effect of nutritional or hormonal interventions on the aging process.

Because aging is probably the cumulative product of multiple basic mechanisms, it is crucial that multiple functions be examined (Schneider, *et al.*, 1982); however, relatively few studies to date have done so. One possible approach to this problem is to measure large numbers of components in biological fluids; this would allow monitoring complex relationships among the metabolites and would also, because of its non-invasive nature, permit longitudinal studies of individual animals.

This approach has been used recently by biochemists to study other complex processes such as diabetes mellitus (Niwa and Maida, 1981; Alasandr, *et al.*, 1982) and neuroblastoma (Gates, *et al.*, 1978) and is called "metabolic profiling" (Horning and Horning, 1971). The most noteworthy application of this technique in aging research comes from Robinson and his coworkers, who used capillary gas chromatography to correlate substances in urine vapor with chronological age of mice (Robinson, *et al.*, 1976). However, it may be more useful to examine the compounds involved in intermediary metabolism rather than the more volatile substances.

During the past several years, we have developed a new metabolic profiling system based upon a high performance liquid chromatograph (HPLC). This system incorporates multiple detectors, computer-based data collection and quality control, and statistical

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techniques to provide reproducible metabolic profiles of a variety of biological fluids. The study described herein utilizes this system to measure the profiles of rats of different ages in order to discover what low molecular weight substances in the urine change with age.

MATERIALS AND METHODS

Sixteen male Fischer F344 rats (four each of 6-, 14-, 23-, and 31-month-old) were obtained from the animal colony maintained by the National Institute on Aging by the Charles River Breeding Laboratory. These rats were barrier reared, and the survival and pathology associated with aging in these rats has been determined (Coleman, *et al.*, 1977). These rats were maintained in our facility in a pathogen-free laminar flow chamber for at least one month before they were used for the experiments described below. The rats were maintained on a 12-hour light/dark cycle and were given Purina Chow and water *ad libitum*. The rats used in these experiments showed neither a loss of body weight, which can be an indication of pathological changes, nor any gross observable lesions.

Twenty-four-hour samples of rat urine were collected by connecting the bottom of the funnel-shaped base of metal metabolic cages to a piece of silanized glass tubing, which in turn led to a small flask kept in dry ice. Thus, the urine was frozen immediately upon release from the animal. The samples were stored at −80°C until analyzed.

The organic acids in the urine samples were extracted using DEAE-Sephadex anion exchange chromatography, as previously described (Rehman, *et al.*, 1982). Aliquots of 2.0 ml were adjusted to pH 7.5 with sodium carbonate, and applied to an 8 cm × 1 cm column of DEAE-Sephadex which had been equilibrated with 0.5 M pyridinium acetate. The neutral and basic substances were eluted with 50 ml water and discarded. The acids were eluted with 30 ml of 1.5 M pyridinium acetate into silanized containers and then lyophilized to dryness.

Each urine sample was reconstituted in 0.50 ml of water, and a 0.020 ml sample was injected into an HPLC column. The HPLC system consisted of a dual-pump gradient-elution system utilizing a 5 micron particle size Ultrasphere ODS 25 cm column with a 5 cm Ultrapak guard column, both maintained at 50°C. The mobile phase, flowing at 2.0 ml/min, consisted of a concave gradient from 0.0% to 40% HPLC-grade acetonitrile against a 0.2M, pH 2.1 phosphate buffer. This system has been carefully optimized to give the best separation of urinary organic acids (Mattiuz, *et al.*, 1982).

The compounds were detected using a triple-detector system. This included a variable-wavelength UV detector (Beckman 155-10), a filter UV detector (Beckman 330), and an electrochemical detector (Bioanalytical Systems) using a glassy carbon working electrode and a Ag/AgCl reference electrode. Data were collected utilizing an LSI 11/23 computer (Digital Equipment Corporation) at a rate of 20 Hz for each detector. Peak areas were integrated using the same computer system and software developed in this laboratory; data from each of the three detectors were treated separately. The output from the computer system thus included integrated peak areas for up to 80 peaks on each of three detectors for each sample.

Corresponding peaks from each sample were then matched by hand. Criteria for the inclusion of a peak for statistical analysis were as follows: a) the peak was adequately resolved from neighboring peaks so that it could be accurately quantified, b) that it not go off-scale, and c) the peak did not appear in the blank samples. Even very small peaks were included as long as they met these three criteria. Computer files of the matched peaks were then used for all of the statistics.

Statistical analyses were all performed using the BMDP statistical package (Dixon and Brown, 1979).

RESULTS

Six types of studies were undertaken: choice of optimum detector parameters; analytical variability; variations of a given rat over a period of several days; differences among rats of the same age; univariate relationships of profile components to chronological age; and multivariate relationships to age.

Optimum detector parameters

Up to 80 components were observed in each urine sample after anion exchange and analysis by the HPLC. The exact number, and the degree of overlap, depended upon the wavelength or electrochemical potential chosen. Wavelengths from 210 nm to 320 nm were chosen for testing, based on a UV spectrum of unextracted urine. In addition, electrochemical potentials from 0.4 V to 1.3 V were compared. In order to obtain the most in-

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formation about the largest number of peaks, wavelengths of 280 nm and 320 nm and an electrochemical potential of 1.0 V were monitored for all samples studied; 280 nm gave the largest number of peaks and hence was used for subsequent statistical studies. A typical chromatogram showing the peaks that were integrated is shown in Figure 1.

Analytical variability

Components of the analytical system used in this study that would contribute to the overall variability include the extraction process, the analysis on the HPLC, and the in-

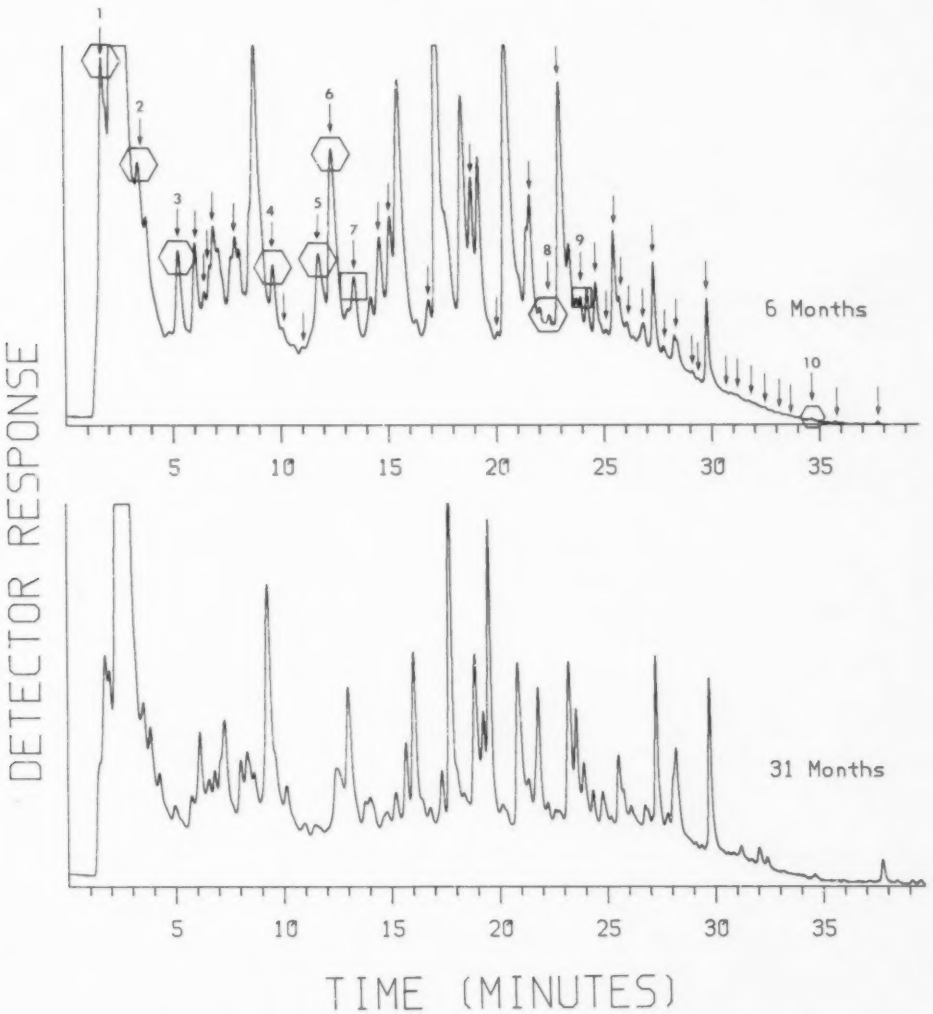

FIG. 1. HPLC profiles of the urinary acids from rats of different ages. The 42 peaks used for statistical purposes are indicated by arrows. The position of the significantly different peaks are shown in hexagons or boxes; boxes show two peaks that were merged for statistical purposes. Peak numbers correspond to those in Table 1.

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tegration by the computer. The median coefficient of variation (relative standard deviation) from these sources was computed by taking the same sample of urine, dividing it into three equal aliquots, and running each sample through the entire extraction and analysis process. The coefficient of variation was then computed for each of the 42 peaks that met our criteria for inclusion (see above), and the median was found to be 13 percent.

Intra-individual variability

One measure affecting the reliability of the observed results is the day-to-day variation in the substances excreted by an individual rat. The median coefficient of variation of intra-individual variation for four samples taken several days apart from a single 18-month-old rat was 25% for the 42 peaks studied.

Inter-individual variability

A limiting factor in most studies on animal metabolism is the inter-individual variability. In typical cross-sectional studies, large variations observed among individuals may mask small but biologically significant changes within a single individual. In a comparison of 42 substances from the urine of four rats of the same age, the median coefficient of variation was 22%; that is, there was no more variation among samples from different individuals than there was among samples from the same individual taken several days apart. This is probably a reflection of the high degree of genetic homogeneity in the rats used.

Univariate comparisons of various ages

Earlier studies (Dirren, *et al.*, 1975) showed that often there are sample-to-sample variations that appear to affect all compounds equally. These variations presumably arise from differences in the animals' fluid uptake and excretion. Therefore, these differences may need to be eliminated in order to be able to perceive small differences related to the aging process. Hence, in addition to using the "unnormalized" data, we also used two different types of "normalization" techniques to eliminate factors affecting all compounds in a sample equally. The details of the normalization techniques are described in the Appendix.

For this study, 16 urine samples were analyzed from 6-, 14-, 23-, and 31-month-old rats. Of the peaks observed, 42 were judged to be present in urine from all animals, and were sufficiently resolved that accurate integration could be achieved. The BMDP One-Way Analysis of Variance test (P7D) was applied to the data, both unnormalized and normalized, and 10 of the 42 peaks were found to change significantly ($p < 0.05$) with the age of the rats; these results are summarized in Table 1. Figure 1 shows a comparison of the metabolic profiles of two of the 16 rats, including the positions of the 10 peaks that change significantly with age.

Graphs illustrating the variation of each statistically significant chromatographic peak with age are shown in Figure 2. The numbering of each graph corresponds to the peaks labeled in Figure 1 and Table 1. The data plotted are the peak areas "normalized to the mean," as described in the Appendix. Also included for each age group on each graph is an error bar showing one standard error on each side of the mean.

It should be noted that peak 10, and to a lesser extent peak 8, show a much higher variability in the oldest groups of rats than in the younger groups. Statistical tests for the

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TABLE 1. ONE-WAY ANOVA PROBABILITIES.

Peak Number	*Unnormalized*	*Normalized to Means*	*Normalized to Sums*
1	0.00	0.00	0.00
2	0.00	0.01	0.08
3	0.02	0.00	0.05
4	0.01	0.01	0.03
5	0.02	0.02	0.03
6	0.00	0.01	0.02
7	0.04	0.00	0.00
8	0.04	0.02	0.01
9	0.01	0.00	0.00
10	0.05	0.04	0.04

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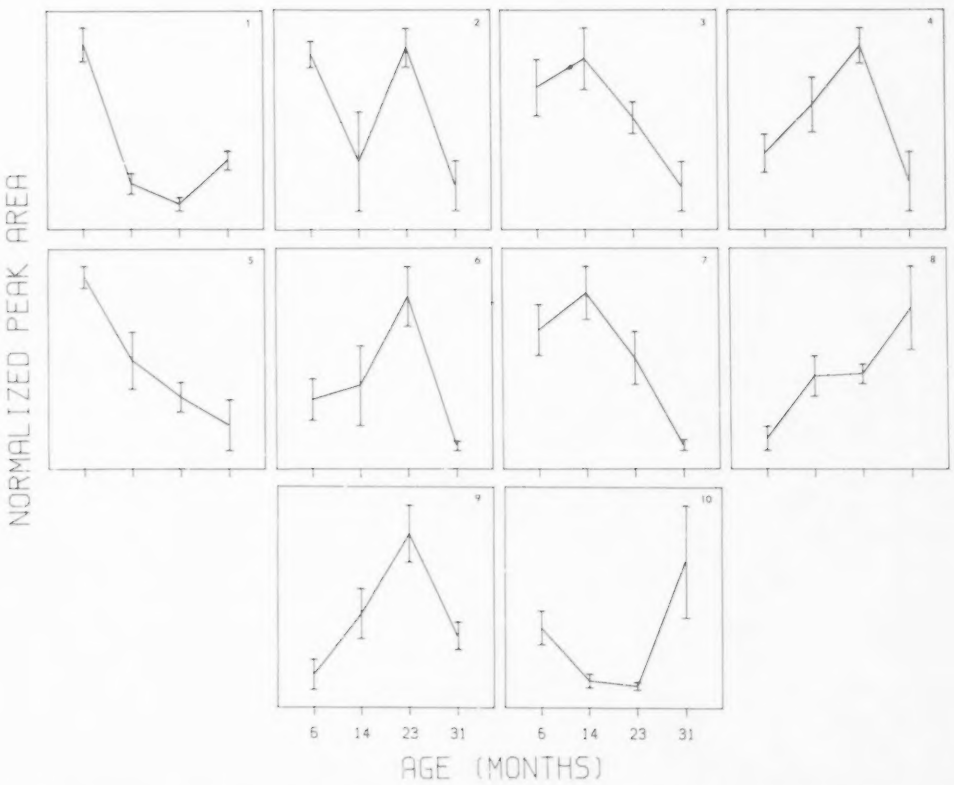

FIG. 2. Variation of the normalized areas of peaks statistically associated with age. Only peaks with $p < 0.05$ in one-way analysis of variance are shown. Peak numbers correspond to those in Table 1 and Figure 1.

equality of variances among the four age groups showed that both peaks 8 and 10 exhibited significantly different ($p < 0.05$) within-group variances, regardless of the type of normalization of the data. Additional analysis of variance tests that do not assume the equality of variances in each age group were computed; both peaks were still generally found to vary significantly with age.

Multivariate relationships with age

Stepwise discriminant analysis was used to determine whether each age group of rats could be distinguished from the others based upon the complete set of 42 measured peaks in the 16 samples described above. Using data normalized to the mean (see Appendix), 8 of the 42 peaks were selected for inclusion in three canonical variables. The first two canonical variables accounted for over 99% of the total dispersion; they are plotted in Figure 3. A jacknifed classification procedure was used to judge the ability of the

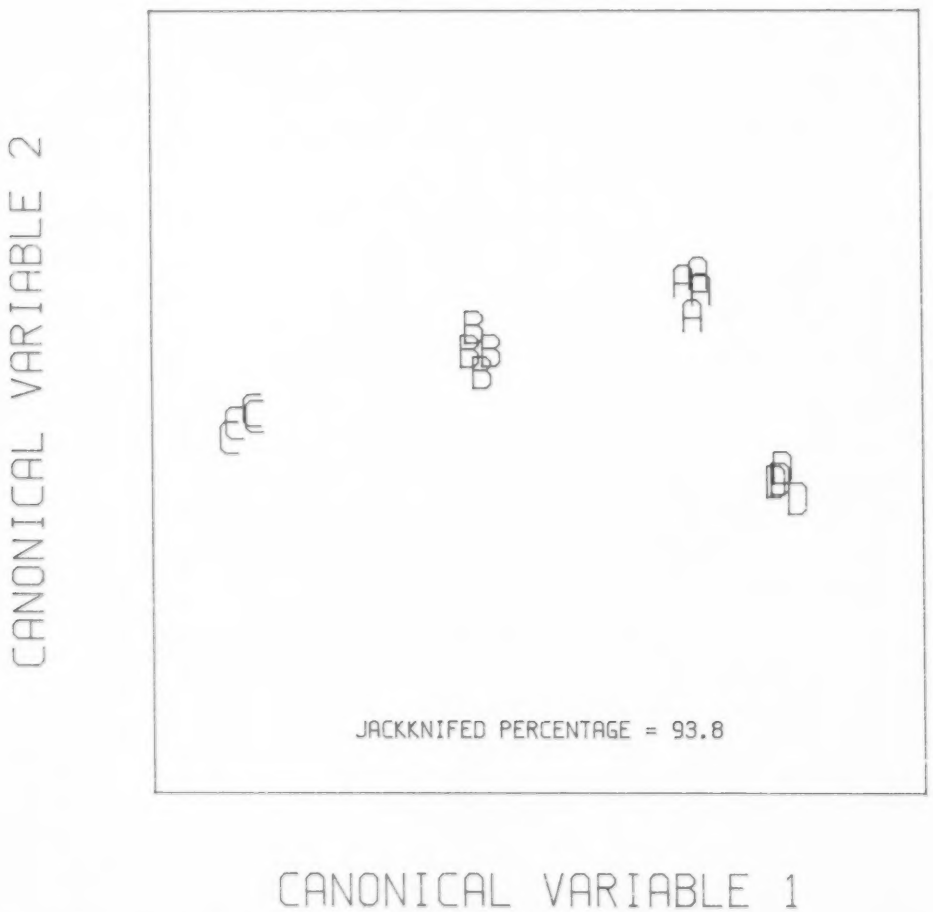

FIG. 3. Stepwise discriminant analysis of 16 rat urine samples. Symbols A = 6 months, B = 14 months, C = 23 months, D = 31 months of age. Canonical variables are derived from 8 of 42 chromatographic peaks measured.

canonical variables to separate the rats in the four age groups. In computing the group means and cross products during the jacknife procedure, each urine sample was eliminated in turn, and the posterior probability computed for the distance of the case to the groups formed by the remaining urine samples (Dixon and Brown, 1979). The overall jacknifed procedure correctly grouped all but one of the 16 urine samples.

DISCUSSION

Studies using quantitative metabolic profiling require both sophisticated instrumentation and very careful attention to proper analytical techniques. In our laboratory, we have found that two innovations are essential for accurate metabolic profiling: a computer-based integration system and automated quality control.

Computer-based data collection and analysis system. Most HPLC and gas chromatographic systems use commercially-available integrators. However, these integrators are generally not designed to handle chromatograms of the complexity of those routinely encountered with the urine samples (see, for example, Figure 1). Hence, we have developed a system that automatically integrates peaks. One of its key features is that the integration is done post-run, rather than real-time (as an integrator must do); therefore, the baseline and peak integration limits can be determined more accurately, utilizing data from the entire run. A second key feature is that the operator can override any computer baseline selection, and cause peaks to be reintegrated; this allows corrections in the rare cases where the computer-chosen baseline appears to be incorrect. A third important feature is that the integrated peak areas can be directly passed to programs that perform statistical analyses, as desired.

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Automated quality control. One of the primary requirements of a system for quantitative metabolic profiling is that it give reproducible results. This is particularly difficult when gradient-elution HPLC is used. We have therefore developed a system which requires that at least one quality control sample be run each day. That sample must pass stringent quality control standards programmed into the computer before the samples can be run for that day. We use an eight-component sample of organic acids as the quality control sample, and judge each of the components based upon the variation of its retention time, peak area, peak width, peak asymmetry and relative peak area. Tolerances are set to be very small, so that even minor variation can cause a standard to be rejected by the computer. In addition, summary data can be plotted for a number of standards analyzed over several months or years, to examine trends in the data.

Using this system, the first objective of this study was to determine the conditions necessary to achieve the best reproducibility and sensitivity. Because the extraction technique had been optimized previously (Rehman, *et al.*, 1982), the major variable to be determined was the best detector to use. UV detection at 280 nm was judged to provide the best sensitivity to a wide variety of substances, while still permitting them to be reasonably well resolved from neighboring components. Because the extraction technique results in the isolation of the organic acids, detection at 280 nm produces a profile of compounds that are almost all aromatic organic acids. Many of these acids have been identified in previous studies (Gates, *et al.*, 1978).

In order to achieve the maximum sensitivity to small differences between different age

groups, it is important that the several sources of variability in the data be minimized. The major advantage of the DEAE-Sephadex anion exchange procedure is that it is highly reproducible. Assuming that the analytical variance is independent of the rat-to-rat variance and that the overall variance is therefore the sum of the contributing variances, about one-fourth of the variance observed in these studies is a result of the analytical variability. Hence, further improvements in the analytical technique will probably not significantly improve the ability to distinguish differences among the different age groups. Indeed, because all rats were highly inbred and the environmental conditions were maintained as uniform as possible, it is probably not reasonable to expect any further improvements in the overall variances either.

Once the possible sources of variance in the data were examined and minimized, a group of 16 rats was studied. One-way analysis of variance was used to discover which compounds in the metabolic profile data varied significantly with age. Surprisingly, differences in almost one-fourth of the peaks were found to be significantly ($p < 0.05$) associated with the chronological age of the rats. The most interesting feature of these results, as shown in Figure 2, is that only two of the ten peaks show a linear trend (peaks 3 and 8). The remaining peaks show a minimum or a maximum at "middle age." This type of trend has been observed in other studies (Dubina, *et al.*, 1983). In the Fischer rats studied, body weight was maximum between 14 and 23 months of age; hence, it is possible that the changes in some of the peaks may be related to changes in body weight rather than the age of the animals.

If the peaks are to be used as markers for the aging process, it should be possible to distinguish one age group from another using multivariate techniques. Multiple regression has been used by at least one group in studying the aging process (Dubina, *et al.*, 1983); however, discriminant analysis offers a graphical method that is easier for the non-statistician to understand. As shown in Figure 3, individual rats can be separated into the individual age groups based upon "canonical variables," which are constructed from linear combinations of the measured variables. In the case of Figure 3, the canonical variables are constructed from 8 of the 42 peaks; 4 of these are among the ten statistically significant peaks shown in Figure 2. It is interesting to note that canonical variable 1 shows a pattern which minimizes at age 23 months (group C), while canonical variable 2 shows a pattern that decreases roughly linearly with increasing age. The four age groups are clearly separated, and individual rats can be assigned to each age group on the basis of the canonical variables with considerable confidence, which is reflected by the jacknifed percentage of over 93%. It would be useful, however, to test this procedure on a much larger group of animals.

It is clear from these results that HPLC-based metabolic profiling represents a viable method for discovering new markers for the aging process; approximately 10 potential markers were found in this study.

Thus, used in combination with modern statistical techniques, as well as careful attention to analytical details, HPLC-based metabolic profiling may prove to be an extremely powerful tool in discovering new markers for the aging process. It may prove to be especially useful as a non-invasive technique for conducting longitudinal studies of the aging process.

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APPENDIX

Three types of data were analyzed: "unnormalized data," which were the peak areas reported by the computer, and "data normalized to sums" and "data normalized to means." The latter two were computed according to the following formulae; in each set of formulae, it is assumed that there is an array of m samples and n unnormalized peak areas, A_{ij}, for each sample, where i is the compound number and j is the sample number.

Normalization to sums

Calculate the sum of all areas for sample j:

$$S_j^{\bullet} = \sum_{i=1}^{m} A_{ij} \tag{1}$$

For the jth sample, the normalization factor is:

$$F_j = \sum_{j=1}^{n} S_j \Big/ S_j \times n \tag{2}$$

The normalized area then is the unnormalized area times the factor for that urine sample; this simplifies to

$$A'_{ij} = \frac{A_{ij}}{n} \frac{\sum_{j=1}^{n} \sum_{i=1}^{m} A_{ij}}{\sum_{i=1}^{m} A_{ij}} \tag{3}$$

Normalization to means

Calculate the mean area for compound i in all of the samples:

$$\bar{A}_i = \frac{\sum_{j=1}^{n} A_{ij}}{n} \tag{4}$$

For each peak, calculate what percent it is of the mean area for that peak:

$$\%_{ij} = 100 \times A_{ij} / \bar{A}_i \tag{5}$$

For each sample, j, calculate the mean of all percents for that sample:

$$\overline{\%}_j = \frac{\sum_{i=1}^{m} \%_{ij}}{m} \tag{6}$$

Then the factor for each sample becomes,

$$F_j = \frac{\sum_{j=1}^{n} \overline{\sigma_0}_j}{\overline{\sigma_0}_j \times n} \tag{7}$$

The normalized area is then the unnormalized area times the factor for that urine sample; this simplifies to:

$$A'_{ij} = \frac{m \times A_{ij}}{n} \frac{(1)}{\sum_{i=1}^{m} \frac{A_{ij}}{\sum_{j=1}^{n} A_{ij}}} \tag{8}$$

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EFFECTS OF U.V. RADIATION ON THE LIFESPAN OF THE ROTIFER *ASPLANCHNA BRIGHTWELLI*

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Abstract—Groups of rotifers of the species *Asplanchna brightwelli* were exposed to U.V. irradiation at dosages ranging from 50 to 4800 J/m². The lifespan of the rotifers was significantly reduced by exposure to U.V. in the range of 200 to 4800 J/m². A logarithmic decline in lifespan was seen as the U.V. dose increased. Rotifers were most sensitive to U.V. exposure during the prereproductive stage at the beginning of their lifecycle. Exposure of rotifers to visible light following U.V. radiation provided no evidence that photoreactivation could influence lifespan in this species.

INTRODUCTION

IT HAS been known for some time that low dosages of ionizing radiation extend the lifespan of a number of species, including *Drosophila* (Lamb, 1965), mice (Lorenz, *et al.*, 1954) and rats (Carlson and Jackson, 1959). It was also reported more recently that exposure to non-ionizing ultraviolet radiation followed by photoreactivation extends the lifespan of clones of *Paramecium tetraurelia* (Smith-Sonneborn, 1979). It is known that the damage to DNA caused by U.V. radiation can be repaired by photoreactivation (Hart and Setlow, 1974; Smith and Hanawalt, 1969), which stimulates repair enzymes to correct accumulated genetic errors. It is believed that this repair process also corrects random errors due to aging, thus causing an increase in lifespan (Smith-Sonneborn, *et al.*, 1983).

The purpose of the present study was to determine whether U.V. irradiation or photoreactivating light exposure would alter the lifespan of the short-lived rotifer *Asplanchna brightwelli*. This rotifer has a five day lifespan which can be lengthened by low temperature (Verdone-Smith and Enesco, 1982) or by Vitamin E supplementation (Sawada and Enesco, in press). Because of this rotifer's relatively small size (400 × 700 μm), the problem of U.V. penetration is less critical than with most other multicellular organisms used in aging studies. Most previous studies on U.V. radiation and lifespan have been confined to *Paramecium,* to yeast and to cells in culture because of the U.V. penetration problem.

MATERIALS AND METHODS

Culture methods

Cultures of the rotifer, *Asplanchna brightwelli* clone 4B61 (originally obtained from Dr. John Gilbert, Dartmouth College, Hanover, N.H.) were grown as indicated by Enesco and Verdone-Smith (1980) in a Cerophyll preparation with *Paramecium caudatum* and *E. coli*. There were 24 or 30 rotifers in each experimental group.

U.V. exposure

The U.V. light source was a 4 W mercury light (Atomic Laboratories Inc., Berkeley, California) with an output of 10 J/m²/sec at a distance of 15 cm and with a frequency of 253.7 nm. The dosage administered was altered by varying the time of exposure. The dosages applied were 50, 150, 200, 300, 600, 1200 and 4800 J/m².

Sheets of Whatman qualitative #1 filter paper, 4.25 cm in diameter, were cut in half (to facilitate its removal later on) and autoclaved for 15 minutes. Using sterile technique, each piece of filter paper was placed inside a sterile 60 × 15 mm Petri dish. Approximately 200 μl of sterile distilled water was then applied to the filter paper.

Rotifers at 12 hours of age were collected and prepared for U.V. exposure. They were washed twice in sterile distilled water to remove the culture medium. Around 30 rotifers were picked up with a micropipette, at a 40 μl setting (Finnipipette Ky, Helsinki, Finland), and gently spread onto the sterile moist filter paper. U.V. light readily passes through a water layer less than 1 mm. Since the water layer on the surface of the rotifers was calculated to be less than 0.5 mm, the absorption of U.V. due to the water was not taken into consideration. The limited moisture content also served to immobilize the rotifers on the surface of the filter paper during U.V. exposure.

Exposure took place in a totally dark room. Seven sets of rotifers were irradiated with increasing dosages of U.V. light while another set of seven control groups as exposed to a 4 W incandescent light source for lengths of time comparable to the experimental groups. After the appropriate exposure period, 10 ml of fresh *Paramecium* medium was supplied to each of the groups. The rotifers were then transferred into an incubator for 12 hours before the first examination. Total darkness was required throughout to prevent photoreactivation. The temperature was maintained at 19.0 ± 0.5°C and the pH of the medium was between 6.5 and 7.0. There was no noticable loss or death of rotifers due to the treatment procedure.

At the first examination period, the half sheet of filter paper was removed and the rotifers were individually segregated into each of the wells of a 24-well tissue culture dish (No. 76-063-05; Flow Laboratories, McLean Virginia) and supplied with 2.5 ml of culture medium each. The wells were examined every 3 hours for lifespan and fecundity measurements. With each examination period, 1 ml of old medium was replaced with 1 ml of fresh medium to ensure a fresh food supply.

Photoreactivation

After exposure to U.V. radiation at 200 or 300 J/m², as described above, each rotifer group was placed in a 60 × 15 mm sterile Petri dish containing 10 ml of sterile water. A 60 W white light source (No. 31-33-01, Bausch & Lomb Inc., Rochester N.Y.) was used as the photoreactivation source (PRS). Distances of 10 and 20 cm and exposures of 5, 10, 15 and 20 min were used in testing for photoreactivation. This dosage range was similar to that used by Sutherland, *et al.* (1980). Following this exposure to light, rotifers were individually segregated and their lifespan was determined as described above.

Statistical analysis

One-way analysis of variance (ANOVA) followed by a *post hoc* Tukey test or two-way ANOVA were performed as indicated in Sokal and Rohlf (1981) and Brunning and Kintz (1977). Logarithmic regression was performed using the transformation of the regression equation to the logarithmic form (Sokal and Rohlf, 1981).

RESULTS

The mean lifespan, maximum lifespan, and average number of offspring for rotifers exposed to the U.V. dosages of 50, 150, 200, 300, 600, 1200 and 4800 J/m² are presented in Table 1, as compared to control values. Using these data, a logarithmic longevity plot was obtained and is presented in Figure 1. Figure 1 shows that there is a logarithmic decline in the lifespan of the rotifers as the dosage of U.V. radiation increased. The equation of the line can be expressed as age = 9.71 − 2.08 log dose. For the control, the vertical line indicates that there were no differences in lifespan among the control groups that had been

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TABLE 1. DATA ON MEAN LIFE-SPAN, MAXIMUM LONGEVITY AND FECUNDITY FOR *A. BRIGHTWELLI* AT VARIOUS U.V. DOSES AND THEIR RESPECTIVE INCANDESCENT CONTROLS ARE PRESENTED.

U.V. Dose (J/m²)	*n*	*Mean Lifespan ± S.E. (days)*	*Max. Longevity (days)*	*Ave. No. Offspring*
Untreated Control	24	5.69 ± 0.17	7.63	8
50	24	5.89 ± 0.14	6.75	7
50 Control	24	5.90 ± 0.11	7.88	7
150	24	5.31 ± 0.21	6.25	6
150 Control	24	5.58 ± 0.16	7.63	8
200	24	5.31 ± 0.14*	6.38	5*
200 Control	24	5.96 ± 0.16	7.38	6
300	24	4.73 ± 0.24**	6.50	4**
300 Control	24	5.38 ± 0.13	6.75	7
600	24	3.98 ± 0.09**	5.63	1**
600 Control	24	5.23 ± 0.13	6.38	6
1200	30	3.30 ± 0.12**	4.63	0**
1200 Control	30	5.37 ± 0.11	7.25	9
4800	30	1.82 ± 0.07**	3.13	0**
4800 Control	30	5.45 ± 0.19	7.38	7

*Significantly different from untreated control (alpha = 0.01)
**Significantly different from either untreated control or incandescent light control (alpha = 0.01)

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exposed for varying times to incandescent light. Statistical analysis showed that there were no significant differences between the control groups exposed to incandescent light and the untreated control group. These data established that neither the method of immobilization nor the exposure to incandescent light had any effect on the lifespan of the rotifers.

Statistical analysis using the one-way ANOVA followed by a *post hoc* Tukey test showed that the rotifers exposed to 300 J/m² were the first group which showed a signifi-

TABLE 2. THE REPRODUCTIVE PROFILE OF *A. BRIGHTWELLI* EXPOSED TO U.V. RADIATION AT A DOSE OF 300 J/M² IS SHOWN (N = 24).

	Ave. Prereprod. Time ± S.E. (days)	*Ave. Reprod. Time ± S.E. (days)*	*Ave. Postrep. Time ± S.E. (days)*	*Ave. Offspring No. Per rotifer*
Untreated Control	2.31 ± 0.06	3.21 ± 0.16	0.17 ± 0.07	8
U.V. Control	2.38 ± 0.11	2.88 ± 0.21	0.08 ± 0.05	7
Exposed Group	2.30 ± 0.11	2.08 ± 0.23**	0.24 ± 0.09	4*

*Significantly different from both untreated and incandescent control (alpha = 0.01).
**Significantly different from both untreated and incandescent control (alpha = 0.05).

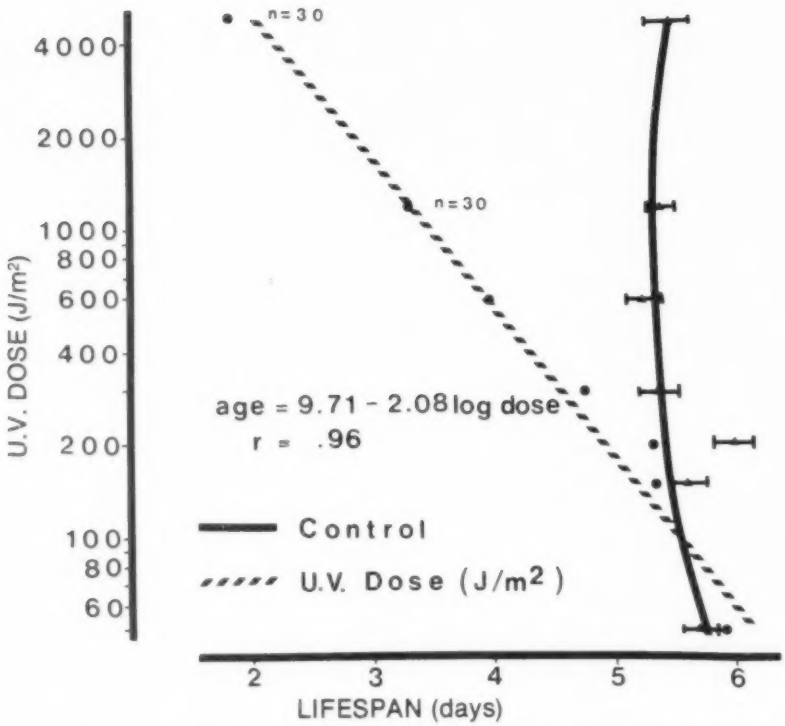

FIG. 1. Logarithmic longevity plot of *A. brightwelli* exposed to increasing dosages of U.V. light (experimental) or incandescent light (control).

cant decrease in both lifespan and fecundity values as compared to both its incandescent light control group and to the untreated control group. For this reason the reproductive profile of the rotifers at the 300 J/m² dose was analyzed. The results are shown in Table 2. To obtain a reproductive profile, the lifespan was divided into three phases: the prereproductive, reproductive and postreproductive period based on fecundity. Neither the prereproductive nor the postreproductive period was altered in length by exposure to U.V. light at 300 J/m². However, the length of the reproductive period was significantly shorter. Table 2 indicates another important point: the number of offspring in this group was also significantly reduced by exposure to U.V. light at 300 J/m².

Next, rotifers at five different stages of their life cycle were irradiated with a dosage of 300 J/m² to determine whether there was a specific developmental period when the rotifer would be most sensitive to U.V. radiation. The results of this study are presented in Table 3. A one-way ANOVA indicated that there were significant differences in the lifespan of rotifers exposed to 300 J/m² at different stages of their life cycle ([F 5, 138] = 9.141, $p < 0.001$). Subsequent *post hoc* Tukey tests showed that the mean lifespan for rotifers exposed at either ½ or 1½ days was significantly shorter than for rotifers irradiated later in life (alpha = 0.01). These data showed the U.V. radiation shortens lifespan to the

TABLE 3. MEAN LIFESPAN OF *A. BRIGHTWELLI* EXPOSED TO 300 J/M² U.V. RADIATION AT DIFFERENT AGE (N = 24).

Age at Exposure (days)	*Mean Lifespan (days)*	*Significance level (alpha)*
½	4.81 ± 0.19	0.01
1½	4.94 ± 0.22	0.01
3½	5.73 ± 0.13	NSD
4½	5.65 ± 0.15	NSD
5½	5.98 ± 0.15	NSD
Control	5.90 ± 0.12	–

NSD—no significant difference

greatest degree when it is applied early in life, during the prereproductive period. U.V. exposure later in life did not reduce the mean lifespan.

To determine whether photoreactivation would influence lifespan, rotifers were exposed to U.V. light of 200 and 300 J/m² and then exposed to visible light at several intensities. The data in Table 4 show that varying the length of exposure to PRS or varying the distance from PRS had no effect on lifespan within the groups of rotifers previously exposed to 200 or 300 J/m².

Table 5 is a summary table which is included to aid in evaluating the question of whether or not photoreactivation acts to repair DNA damage and extend the lifespan of the rotifer. As seen in Table 5, the lifespan of the U.V. treated rotifers and the U.V. plus PRS treated rotifers are nearly identical. Statistical analysis confirms that the mean lifespan of the U.V. and the U.V. plus PRS groups are significantly different from the

TABLE 4. MEAN LIFESPAN OF *A. BRIGHTWELLI* AFTER U.V. IRRADIATION PLUS PHOTOREACTIVATION (N = 24).

	200 J/m² Dosage U.V. Distance of PRS (cm)	
	10	*20*
Length of PRS exposure (min)	*Mean lifespan (days)*	*Mean lifespan (days)*
5	5.32 ± 0.16	5.31 ± 0.21
10	5.31 ± 0.13	5.29 ± 0.12
15	5.29 ± 0.12	5.32 ± 0.17
20	5.33 ± 0.09	5.26 ± 0.22
	300 J/m² Dosage U.V.	
5	4.62 ± 0.12	4.58 ± 0.21
10	4.56 ± 0.11	4.71 ± 0.19
15	4.55 ± 0.21	4.55 ± 0.25
20	4.47 ± 0.24	4.66 ± 0.20

No significant differences

TABLE 5. SUMMARY OF DATA ON LIFESPAN.

	Mean lifespan	
Group	*200 J/m²*	*300 J/m²*
Control	5.96 ± 0.16	5.38 ± 0.13
U.V.	5.25 ± 0.14*	4.57 ± 0.21*
U.V. + PRS (5 min at 10 cm)	5.32 ± 0.16*	4.62 ± 0.12*

*significantly different from control at alpha = 0.05

control, but not different from each other. Thus, there is no evidence that photoreactivation takes place in *A. brightwelli.* There was no increase in the lifespan under the conditions used here.

DISCUSSION

The results presented here show that U.V. radiation at dosages above 200 J/m² shortened the lifespan of *A. brightwelli.* This finding is in agreement with previous studies on the effects of U.V. irradiation on both unicellular and multicellular organisms. Using yeast, Muller and Wolf (1978) showed that increasing dosages of U.V. radiation reduced the lifespan of the yeast as measured by its budding potential. Similarly, Smith-Sonneborn (1971, 1979) has demonstrated that increasing dosages of U.V. radiation reduced the lifespan of clones of *Paramecium* as measured by their division potential and rate of clonal senescense. Working with a multicellular organism, Klass (1977) showed that U.V. radiation shortens the lifespan of the nematode *Caenorhabditis elegans.*

We were initially concerned about the problem of whether U.V. light would penetrate the rotifers. Because of the low penetrating power of U.V. radiation, most studies with U.V. have been done using unicellular organisms such as yeast (Muller and Wolf, 1978) *Paramecium* (Smith-Sonneborn, 1979) or tissue culture cells (Hart and Setlow, 1974). However, it should be noted that the results presented here show that the mean lifespan of the rotifers decreased logarithmically as it is exposed to increasing dosages of U.V. radiation. This pattern of logarithmic decrease in lifespan is identical to the pattern observed when ionizing radiation is used; for example, Sacher and Grahn (1964) showed a similar logarithmic decrease in survival values when increasing dosages of gamma radiation were applied to LAF1 mice. Because this logarithmic pattern was observed, it suggests that U.V. penetration through the rotifer was not a problem in this study.

It is well established that low dosages of gamma radiation can increase lifespan for *Drosophila* (Lamb, 1965) and for mice (Lorenz, *et al.,* 1954). In contrast, any dosage of U.V. radiation shortened the lifespan of clones of *Paramecium* (Smith-Sonneborn, 1979) and of nematodes (Klass, 1977) as it did for rotifers in the present study. The life-shortening effect of U.V. radiation was first apparent in our study at a U.V. dosage of 200 J/m², the third highest dosage used. U.V. radiation at 50 or 150 J/m² had no effect on the lifespan or fecundity of the rotifers.

Both shortening of lifespan and a drop in fecundity are seen for the first time at a dosage of 200 J/m², although the changes are not pronounced. In analyzing the fecundity data presented here, it is noteworthy that the number of offspring of rotifers exposed to 300 J/m² was reduced by 50 percent as compared to controls. This reduction in number of offspring may be accounted for by U.V. damage at two levels: damage to vitellarium which produces yolk for the embryo and damage to the germ cells or to the embryo itself. The vitellarium and the gastric glands are the only two organs which continue to undergo cell division in the adult rotifer (Jones and Gilbert, 1977). Although the effects of U.V. damage to DNA may be similar in dividing and non-dividing cells, the biological consequences would be more far-reaching when the DNA damage interferes with the process of embryogenesis.

Our results further show that young rotifers are more sensitive to U.V. radiation than old rotifers. Similar results were reported for nematodes (Klass, 1979). The cuticle thickens with age in both rotifers and nematodes. The need for cell division decreases as these organisms age since both are composed of post-mitotic cells as adults. In contrast, *Paramecium* become increasingly sensitive to U.V. radiation with age (Smith-Sonneborn, 1971).

U.V. radiation produces a well documented type of damage to DNA, characterized by pyrimidine dimer formation which can be repaired by specific light-activated endonucleases. This process is known as photoreactivation (Hart and Setlow, 1974; Sutherland, *et al.*, 1980). Smith-Sonneborn (1979) has reported that when *Paramecium* are exposed to U.V. radiation and then exposed to photoreactivating light, their clonal lifespan is significantly increased as compared to untreated control *Paramecium*. The interpretation given by Smith-Sonneborn (1979, 1981) of these results is that the age-related DNA damage is repaired by exposure to the U.V. and photoreactivation. In this context we assessed the result of U.V. plus PRS on rotifers. Our results show that there is no difference in the lifespan of U.V. treated rotifers as compared to U.V. plus PRS treated rotifers. Thus we have no evidence that a photoreactivation process exists in rotifers. It may be that the development of the enzymes necessary for the photoreactivating system would not be of adaptative advantage to *A. brightwelli* with its five day lifespan. In comparison, *Paramecium tetraurelia* has a clonal life-span of about 40–60 days (Smith-Sonneborn, *et al.*, 1983).

The degree to which cells from different species can carry out excision repair of DNA damage following U.V. irradiation is thought to be correlated with the lifespan of that species (Hart and Setlow, 1974), although there is conflicting evidence on this point (Woodhead, *et al.*, 1980; Regan, *et al.*, 1982). It is possible that the need for repair is minimal in somatic cells of a short-lived, post-mitotic organism such as the rotifer.

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FOOD INTAKE REDUCTION AND IMMUNOLOGIC ALTERATIONS IN MICE FED DEHYDROEPIANDROSTERONE

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Abstract—A diet containing 0.4% DHEA was fed to male mice of a long-lived strain from 3 weeks until 18 weeks of age. These mice were compared with others fed a control diet *ad libitum* and with mice pair-fed the control diet in amounts approximating the intake of the DHEA-fed group. Mice fed the DHEA diet failed to eat all of the food presented to them whereas the pair-fed mice ate all of their food. All mice were studied at 18 weeks of age for two age-sensitive immune parameters (spleen lymphocyte proliferation induced by T-cell mitogens [PHA or ConA] and natural killer cell lysis of an allogeneic tumor). DHEA feeding led to: 1) a decrease in food intake (~30% less than for mice fed the control diet *ad libitum*), 2) a lower body weight at 18 weeks of age (~40% lower than for *ad libitum* controls) due to a decrease in the body weight gained from 3 weeks through 18 weeks of age (~55% lower than controls), 3) a lower spleen weight (~30% lower than controls) but without lower numbers of nucleated cells per spleen, 4) an increase in PHA-induced proliferation by spleen lymphocytes (~100% higher than for controls) and, 5) no influence on splenic natural killer cell activity. The inhibition of body weight gain for mice fed DHEA appeared due to both a reduction in food intake and a metabolic effect since mice eating DHEA gained less body weight per gram of food eaten than did mice in either group eating the control diet.

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INTRODUCTION

DEHYDROEPIANDROSTERONE (DHEA) AND DHEA-sulfate (DHEA-S) are major secretory products of the adrenal cortex in humans (Van de Wiele and Lieberman, 1960). Plasma levels of DHEA-S peak in humans at ~ 50–100 μg/dl at 20–30 years of age and decline thereafter (Migeon *et al.* 1957; de Moor and Heyns, 1966; Wang *et al.*, 1974; Rose *et al.*, 1977). Women with low urine excretion rates of androsterone and etiocholanolone (both derived from DHEA) experience an increased risk of breast cancer (Bulbrook *et al.*, 1971). Urinary output of DHEA was found by Lopez-S. and Krehl (1967) to be low in obese people and to increase with weight loss; in contrast, Hendrikx and coworkers report (1966) that obese people show high urinary excretion rates for DHEA which fall with weight loss. Although the precise biologic function of DHEA remains unclear, DHEA is a potent non-competitive inhibitor of mammalian glucose-6-phosphate dehydrogenase

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(Marks and Banks, 1960; Oertel and Benes, 1972), one of the enzymes producing NADPH for fatty acid synthesis.

DHEA and DHEA-S are also abundant in sera from the highest non-human primates but are found in very low levels in sera from other adult mammals (Cutler *et al.,* 1978; Winter *et al.,* 1980). For example, Cutler and colleagues (1978) find that serum levels of DHEA and DHEA-S in rats, hamsters, pigs and cows do not exceed detection limits for each assay (25 ng/dl and 5 μg/dl, respectively) versus ~ 500 ng/dl DHEA and 20 μg/dl DHEA-S in sera from adult gorillas. Levels of DHEA-S in rat brain exceed plasma levels by up to 20 fold and do not seem to depend on adrenal secretion (Corpechot *et al.,* 1981).

Anti-obesity, anti-cancer and anti-diabetes effects have been described for mice treated with DHEA. Treatment of A^{vy}/a mice (a genetically obese strain) with certain doses of DHEA reduces rates of body weight (BW) gain without lowering food intake (FI) (Yen *et al.,* 1977). Schwartz (1979) also finds that DHEA-treated A^{vy}/a mice show lower BW but do not eat less food than controls. In addition, he reports that DHEA treatment inhibits formation of spontaneous breast cancer in this highly susceptible strain. One i.p. injection of DHEA into C3H or ICR mice lowers the rate of tritiated thymidine (^{3}H-TdR) incorporation in breast epithelium as well as in epidermis stimulated by tumor promoter (Pashko *et al.,* 1981). Treatment of A/J mice with DHEA in the diet reduces the amount of ^{3}H-7,12-dimethylbenz(a)anthracene bound to mouse skin DNA twelve hours after the carcinogen is topically applied (Pashko and Schwartz, 1983). A/J mice fed DHEA in the diet show reduced BW gain without reduced FI and an inhibition of occurrence of chemically induced lung tumors (Schwartz and Tannen, 1981). DHEA feeding reduces the severity of both non-insulin dependent or insulin-dependent diabetes in C57BL/KsJ mice without decreasing FI (Coleman *et al.,* 1982). Cleary and coworkers (1982) find that C3H mice treated with DHEA show lower BW, lower weights of epididymal and retroperitoneal fat pads, and smaller fat cells than controls; however, FI and fat cell numbers were not influenced by DHEA.

We report now on influences of dietary DHEA on BW, FI and age-sensitive immune functions in a long-lived mouse hybrid strain.

MATERIALS AND METHODS

Mice

A long-lived F_1 hybrid strain was studied. The mean lifespan for females of this F_1 hybrid is ~33 months (unpublished observation). Females from the C3H.SW/Sn inbred strain were mated with males from the C57BL10.RIII/Sn inbred strain in our animal facilities. Male hybrid progeny were weaned at 21–24 days of age and individually caged in plastic cages. Two folded paper towels were placed on the cage bottom (instead of wood bedding) to aid in determination of in-cage food waste.

Diet groups

Six mice were randomly assigned to each of three test groups at weaning: 1) Control diet, *ad libitum;* 2) Control diet, pair fed to the FI of mice in Group 3; and 3) Control diet supplemented with DHEA (G.D. Searle, Skokie, IL) to a level of 0.4% (by weight). Pilot food intake studies of mice fed the DHEA diet provided guidelines for the amount of diet fed to Group 2. Both diets were kindly provided by Dr. Arthur Schwartz and consisted of Purina Lab Chow repelleted by Bioserv (Frenchtown, N.J.) to include 3% acacia to yield a harder pellet. Body weights were recorded at weaning and on each Friday morning thereafter.

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Feeding strategies

Mice in Group 1 were given 100 g of food every Friday. Mice in Groups 2 and 3 were fed a preweighed amount of food on Mondays, Wednesdays and Fridays. The amount of food given to each mouse was 17.5 g/wk for weeks 1 and 2, 21.0 g/wk for weeks 3 and 4, 24.5 g/wk for weeks 5–8, 22.5 g for week 9, 21.0 g for week 10, 27.0 g for week 11 (mistakenly overfed), and 21.0 g/wk for weeks 12–15. The amount of food given was reduced from 24.5 g/wk after week 8 to 21.0 g/wk at week 10 because mice on the DHEA diet were not eating that amount of food whereas Group 2 mice ("pair fed") ate all food given them. FI for each mouse was determined each Friday by weighing the food remaining in the cage and in the hopper.

Immunologic tests

Mice were killed at 18 weeks of age by cervical dislocation. The spleen was asceptically removed, weighed, placed in cold media (RPMI-1640 [Microbiological Associates, Los Angeles, CA]) supplemented with pencillin, streptomycin, fungizone and 25 mM HEPES buffer. Only individual spleens were studied (no pooling). Each spleen was gently pressed through a sterile aluminum mesh screen with a tuberculin syringe plunger. The cell suspensions were washed once and erythrocytes lysed with 7% ammonium chloride. The suspensions were then washed three times in media. Cell viability ranged from 80–95% as judged by trypan blue exclusion.

About one-half of each spleen cell suspension was used to test for *in vitro* proliferative responses to the T-cell mitogens phytohemagglutin (PHA) and concanavalin A (ConA) using a microculture method (described in Weindruch *et al.*, 1982a) to assay mitogen-induced DNA synthesis. Mitogen doses used (2.5 μl/ml for PHA, 2.0 μg/ml for ConA) were found in preliminary studies to optimally stimulate spleen cells from 3–4 month old (C3H.SW/Sn × C57BL10.RIII)F_1 mice. Triplicate cultures were carried out for each mitogen-stimulated and control test. Optimal incubation time was 48 hours. Cultures were labelled the last 24 hours with 2 μCi of ^{3}H-TdR (1.9 C/M mole activity). Results are reported as (counts per minute [cpm] in stimulated cultures) − (cpm in control cultures).

The other portion of each spleen cell suspension was tested for natural killer (NK) cell activity against the YAC-1 tumor (a Moloney virus-induced T-cell lymphoma from A/Sn mice). NK response was measured at various effector: target ratios in a 4-hour ^{51}Cr-release assay (Gupta *et al.*, 1978) and the percent of cytotoxicity calculated as (experimental release-spontaneous release)/(maximum release-spontaneous release) × 100.

Statistics

Intergroup differences were evaluated via the two tailed *t* statistic for two means.

RESULTS

Body weight & food intake

Influences of dietary DHEA on body weight (BW) gain and food intake (FI) over the course of the experiment are shown in Figure 1. DHEA-fed mice (Group 3) ate less food and gained less BW than did mice given free access to the control diet (Group 1). Mice fed the DHEA diet tended to scatter food on the cage bottom whereas mice on the control diet did not show this behavior. Examination of Table 1 shows that BW at the end of the study (18 weeks of age) for Group 3 mice averaged ~40% less than those for Group 1. The change in BW from weaning to 18 weeks of age (ΔBW) was ~55% lower for Group 3 than for Group 1. Total FI was ~30% lower for Group 3 mice compared to Group 1. The "pair fed" controls (Group 2) were not pair fed since their total FI was slightly more (~10%) than that for Group 3. This occurred as a result of Group 3 mice eating less food than we expected and caused us to reduce the food allotted to mice in Groups 2 and 3 during the study (as explained above). The index ΔBW/total FI provides an estimate of how efficiently food was converted to BW and was ~30% lower for Group 3 than for Groups 1 and 2.

TABLE 1: INFLUENCE OF DIETARY DHEA ON BODY WEIGHT & FOOD INTAKE

	Body Weight (g)			*ΔBody Weight*
Diet	*@ 3 wks.*	*@ 18 wks.*	*Total Food Intake*	*Total Food Intake*
1. Control, ad lib	12.5 ± 1.0	43.0 ± 2.0	413 ± 14	0.074 ± .004
2. Control, pr. fed	12.5 ± 1.0	35.0 ± 1.5	320 ± 3	0.069 ± .003
3. DHEA, ad lib	12.0 ± 0.5	26.0 ± 0.5	290 ± 6	0.049 ± .002

Values are $\bar{X}$ ± SEM for 6 mice per diet group. Feeding began at weaning (21–24 days old) and lasted until 18 wks of age. Statistical comparisons: For body weight @ 18 wks−1 > 2 ($P < .01$) or 3 ($P < .001$). 2 > 3 ($P < .001$). For total food intake−1 > 2 or 3 ($P < .001$), 2 > 3 ($P < .01$). For Δbody weight/total food intake−1 or 2 > 3 ($P < .001$).

Spleen size & cell yield

Influences of dietary DHEA on spleen wet weight and on numbers of nucleated cells per spleen are shown in Table 2. Spleen weights for mice fed DHEA were ~30% lower than for Group 1 controls. Spleen weights for Group 2 mice were ~25% lower than for those of Group 1 controls but did not differ significantly from those of Group 3 mice. The spleen weight: BW ratio ranked Group 3 > 1 > 2. Statistically significant diet effects on spleen cell yields were not observed.

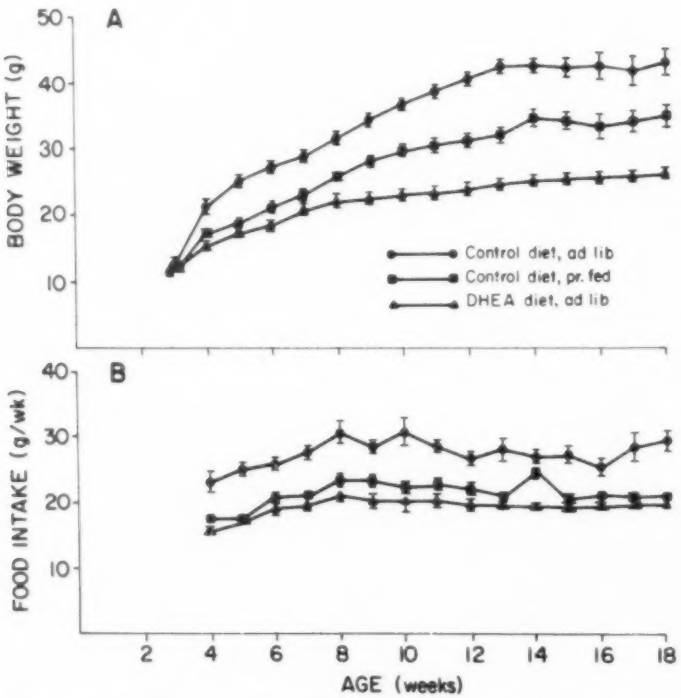

FIG. 1. Body weight gain (Panel A) and food intake (Panel B) for mice fed DHEA and for controls. Values represent $\bar{X}$ ± SEM for 6 mice per diet group.

TABLE 2: INFLUENCE OF DIETARY DHEA ON SPLEEN WEIGHT AND NUMBERS OF NUCLEATED CELLS PER SPLEEN.

Diet	*Spleen Wt. (mg)*	*Spleen Wt. (mg)/BW (g)*	*Cells/Spleen (× 10⁶)*
1. Control, ad lib	116 ± 6	2.7 ± 0.1	78 ± 16
2. Control pr. fed	88 ± 4	2.3 ± 0.1	65 ± 8
3. DHEA, ad lib	84 ± 2	3.1 ± 0.1	63 ± 8

Values are $\bar{X}$ ± SEM for n=6. Statistical comparisons: For spleen wt.—1 > 2 ($P < .01$) or 3 ($P < .001$). For spleen wt./BW—1 > 2 ($P < .01$), 3 > 1 ($P < .01$) or 2 ($P < .001$). For cells/spleen—no significant differences.

TABLE 3: INFLUENCE OF DIETARY DHEA ON SPLENIC LYMPHOCYTE PROLIFERATION AND NK ACTIVITY.

	Lymphocyte Proliferation			*NK*	
Diet	*PHA*	*Con A*	*Media*	*100:1*	*25:1*
1. Control, ad lib	15,500 ± 3,625	52,575 ± 14,200	500 ± 100	23 ± 2	15 ± 2
2. Control, pr. fed	21,975 ± 4,750	50,150 ± 15,025	575 ± 150	21 ± 2	11 ± 1
3. DHEA, ad lib	30,850 ± 3,425	83,050 ± 7,425	1,450 ± 200	21 ± 1	13 ± 1

Values are $\bar{X}$ ± SEM (n= 6). Mitogen data are expressed as cpm of ^{3}H-TdR in mitogen stimulated cultures minus cpm in unstimulated (media without mitogen) cultures. NK data represent percent specific lysis of YAC-1 targets in a 4-hr ^{51}Cr release assay. Statistical comparisons: For PHA—3 > 1 ($P < .02$). For ConA—no significant differences. For Media—3 > 1 or 2 ($P < .01$). For NK—no significant differences.

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Immunologic tests

Spleen cell proliferation in response to PHA or ConA as well as NK response to the YAC-1 lymphoma were measured (Table 3). Mice on the DHEA diet showed higher responses ($p < .02$) to PHA then did mice in Group 1. PHA responses did not differ significantly between mice on DHEA and mice in Group 2. ConA responses were not significantly influenced by diet. Spleen cells from mice on the DHEA diet incubated in media without PHA or ConA incorporated significantly more ($p < .01$) ^{3}H-TdR than was found for splenocytes of mice in Groups 1 and 2. NK responses were not significantly influenced by diet.

DISCUSSION

The present results indicate that feeding a diet containing DHEA to mice from 3 weeks through 18 weeks of age leads to: 1) a decrease in FI (~30% less than for mice in Group 1), 2) a lower BW at 18 weeks of age (~40% lower than for Group 1 mice) due to a decrease in the post-weaning BW gain (~55% lower than for Group 1 mice), 3) a lower spleen weight (~30% lower than for Group 1 mice) but without an effect on numbers of nucleated cells per spleen, 4) an increase in PHA-induced proliferation of spleen lymphocytes (~100% higher than for Group 1 mice) and, 5) no influence on NK activity.

Our results show that feeding DHEA to young, growing male mice can strikingly inhibit body weight gain. This inhibition appears to occur by both a reduction in voluntary FI (~30%) and via a metabolic effect. A similar reduction in food intake was observed by us

in two earlier DHEA feeding studies (Weindruch *et al.*, unpublished observations) and led us to include a pair-fed group in the present experiment. A metabolic effect is suggested by the finding that mice on the DHEA diet gained 0.049 ± .002g of BW/g of FI during the 15 weeks of feeding. This value is ~35% lower than the 0.074 ± .004g BW/g of control diet eaten on an *ad libitum* basis. For Group 2 mice, the value is 0.069 ± .003g BW/g FI. Thus, mice given free or restricted (75–80% of *ad libitum*) access to the control diet appear to convert consumed food into BW more efficiently (~30–35%) than do mice eating a DHEA-containing diet.

Only very few prior reports describe lower FI for rodents fed DHEA. Yen and coworkers (1977) report that A^{vy}/a mice eating a 0.2% DHEA diet gain less BW but eat the same amount of food as do mice on a control diet; however, mice on a 0.5% DHEA diet "did not eat well" (but data were not presented). Further, treatment of A^{vy}/a mice with DHEA as 500mg/kg, p.o., three times weekly leads to an anti-obesity effect (e.g., at 17 weeks of age control males weigh ~35g vs. ~25g for DHEA-treated mice), in the face of an *increased* food intake (4.7 g/mouse/day for DHEA-treated mice vs. 3.8 g/mouse/day for controls). Schwartz (1979) put female C3H(A^{vy}/a) mice on a similar treatment protocol (450 mg/kg, p.o., three times weekly). He finds that BW gain is clearly inhibited by DHEA treatment (e.g. at 21 weeks of age, control BW average ~50g vs. ~35g for DHEA-treated mice) but FI is not influenced. Pashko and Schwartz (1983) began treatment (0.6% DHEA in diet) on 5–6 week old male A/J mice. They report, a sharp fall in FI for mice on DHEA during the first 2 weeks of the experiment (~60% of control level for week 1, 30% for week 2). Over the final 8 weeks the FI for DHEA-treated mice rose to a level ~8% greater than for controls. Average FI over the whole study was not significantly influenced by DHEA feeding. Final BW averaged ~25g for controls and ~20g for mice fed DHEA. Schwartz and Tannen (1981) put six week old male A/J mice on diets with 0, 0.2, 0.4 or 0.6% DHEA for 20 weeks. They report final BW for these respective groups as approximately 33g, 27g, 24g and 20g. Total FI did not differ among these groups despite an initial two week depression in FI at the higher DHEA doses. Coleman and colleagues (1982) report that genetically diabetic C57BL/KsJ mice on a 0.4% DHEA diet eat ~6g of food per day versus ~4g for controls. Yet, the rate of BW gain was slower for mice eating DHEA. Cleary and coworkers (1982) report that male C3H mice given DHEA (450mg/kg p.o.) from 6–16 weeks of age show a slightly lower final BW (27.1g vs. 30.4g) but no effect on FI. This group also reports (Cleary *et al.*, 1983) that 0.3–0.6% DHEA decreases BW gain but not FI in growing Sprague-Dawley rats. Further, DHEA feeding strikingly increases liver malic enzyme activity. Feeding a 0.6% DHEA diet to lean Zucker rats from 12–18 months reduces neither BW nor FI but increases liver malic enzyme and acyl CoA hydrolase activities (Cleary *et al.*, in press). One very recent report (Nyce *et al.*, 1984) does accord with our findings as 2–3 month old female Balb/c mice fed a 0.6% DHEA diet for 10 months eat only ~75% as much food as do controls. BW gain occurred only in the controls (from ~17 to 23 g). Tannen and Schwartz (1982) find that feeding female NZB mice a 0.4% DHEA diet from 2–6 months of age lowers final BW (30 vs 44g) without influencing FI. Tagliaferro and Davis (1983) observe that 0.6% DHEA fed to Sprague-Dawley rats for 2 months reduces BW gain by increasing resting metabolic rate (without influencing FI). Thus, almost all prior reports differ from the present one in not describing a strong and sustained reduction in FI in mice fed DHEA. Possible explanations for our discordant finding include the strain of mouse, the feeding strategy, and the method used to measure FI.

Clearly, any potential anti-aging actions of DHEA treatment must be separated from the well-documented anti-aging effects of lifespan-prolonging dietary restriction (see Barrows and Kokkonen, 1982; Bertrand, 1983; and Weindruch *et al.*, 1979 for reviews of these effects). The 30% fall in FI which we now report for mice fed DHEA is a less severe diet restriction than is used in most aging studies. For example, we feed restricted mice ~45% fewer calories than are fed to controls (Weindruch *et al.*, 1982a). Masoro and coworkers feed restricted rats 40% less food (Yu *et al.*, 1982). Merry and Holehan (1981) employ a 50% diet restriction.

DHEA is not unique in being a substance which produces both desirable biologic effects and lower body weights in mice. For example, mice fed levodopa (Cotzias *et al.*, 1977) or ethoxyquin (Comfort *et al.*, 1971) live longer but weigh less than controls. FI was not influenced by dietary levodopa and was not measured in mice fed ethoxyquin. The method used to measure FI for mice on levodopa diets was not described. In our view, for studies where rodents are fed a potential anti-aging drug it seems crucial to: 1) very carefully measure FI for treated rodents and, 2) if differences obtain, include a pair-fed group on the control diet.

The present immunologic findings point toward influences of DHEA on mitogen-induced and uninduced lymphocyte proliferation but not on NK responses. The proliferative response of mouse T-lymphocytes to PHA and Con A mitogens falls with age (Hori *et al.*, 1973; Meredith and Walford, 1977) as does NK activity (Roder, 1980; Weindruch *et al.*, 1983). We have previously reported that life-prolonging diet restriction of the same F_1 hybrid strain as was studied here results in: 1) higher PHA responses (Weindruch *et al.*, 1982a,b) and, 2) lower basal NK responses but higher NK responses after injection with the interferon inducer Poly I:C (Weindruch *et al.*, 1983). The mice on DHEA show higher PHA-induced proliferation; however, this obtains only when data are calculated as Δ cpm (i.e. cpm in stimulated cultures minus cpm in control cultures). Lymphocyte proliferation data are occasionally reported as the "stimulation index" (= cpm in stimulated/cpm in control). Calculation of the present data in this way would lead to a different conclusion, namely, that mice on DHEA do not differ from *ad libitum* controls in PHA response. Further, the PHA stimulation index for mice fed DHEA is significantly ($p < .01$) less than that for pair-fed mice on the control diet. We do not favor use of the stimulation index as it appears to over-emphasize any fluctuations in unstimulated ^{3}H-TdR uptake. The higher cpm found in cultures without PHA or ConA with splenocytes from DHEA-fed mice is difficult to explain. In our previous work with mice on restricted diets, we generally do not find influences on cpm in unstimulated cultures (Weindruch *et al.*, 1982a,b). The sole exception is that lymphocytes from restricted mice fasted for 48 hrs. before assay show lower unstimulated cpm than do cells from controls or restricted mice fasted 24 hours (Weindruch *et al.*, 1982a). Finally, it seems noteworthy that spleen size and cell yields appear more clearly reduced by lifespan-prolonging dietary restriction than by DHEA feeding even though both regimes, in our hands, lower BW by a similar proportion.

The present findings underscore the possibility that a decreased FI may contribute to biologic effects found in mice fed diets containing DHEA.

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AGING CHANGES IN THE OPTIC TECTUM OF THE GUPPY *POECILIA (LEBISTES) RETICULATUS*

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Abstract—Histological surveys of the brains of guppies throughout their lifespan showed no overall loss of tissue with advancing age. Brain and body increased in size at a similar rate throughout adult life in male fish. In old females after the age of two years, brain growth apparently ceased, although body growth continued at a low rate. In both sexes there was a loss of neurones from the stratum griseum periventriculare in the midbrain roof in old age; the decrease in size was significant. The midbrain is a major correlative center concerned with spatial orientation. How far neuronal loss may contribute to functional behavioral disorder in old age is unknown, but a loss of orientation could render the older fish more susceptible to predation.

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INTRODUCTION

THE NERVOUS system of mammals has long been a favored subject for researches into aging processes because it consists of highly differentiated postmitotic cells that appear to have a lifespan which goes hand in hand with the longevity of the species (Sacher, 1959). Investigators at the turn of the century had already linked senescence in humans, white rats and insects with morphological deterioration and the loss of about 35 percent of brain tissue (Hodge, 1894; Hatai, 1902; Ellis, 1919, 1920). Recent work has modified these early conclusions showing that there is not a general overall loss of nerve cells from the mammalian brain, but that neuronal loss is highly selective both in space and time, and may vary between species, strains and the sexes (Brody, 1955, 1970; Johnson and Erner, 1972; Henderson *et al.*, 1980; Sable and Stein, 1981; Wexler *et al.*, 1981; McNeill, 1983). Other quantitative changes that have been recorded include a decrease in brain weight and in extracellular space, the extent of which varies with the species (Terry, 1978; McNeill, 1983). Among the qualitative alterations described in the aging brain of mammals are an increasingly inadequate blood circulation, lipofuscin accumulation, damage to cytoplasmic granules, dendritic regression and aberrant dendritic sprouting (Scheibel, 1979; Davies and Fotheringham, 1981, *a, b, c;* Buell and Coleman, 1979, 1980). The distribution of these morphological changes within the brain is not uniform (Brizzee and Knox, 1980).

*Research carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy.

Apart from these studies with primates and rodents, and some detailed observations on aging in the brains of honey bees, houseflies and *Drosophila* (for example, Sohal and Sharma, 1972; Miquel *et al.*, 1979), there have been few comparative studies. This lack of information is understandable. Other than the excellent treatise by Ariëns Kapper *et al.*, (1936), information on the comparative anatomy of the nervous system is sparse and scattered in the literature. Few animals except mammals are kept into old age and even in the well-studied primates and rodents there is uncertainty as to what constitutes an old animal (for instance, different strains of rats have very different lifespans). Further, despite the wealth of histological observations in these two orders, it is sometimes difficult to distinguish between aging changes and pathological lesions in brain tissue that has been preserved by standard histological procedures (Sulkin, 1961).

For some years we have been documenting aging changes in the tissues and organs of the guppy (for example, Woodhead and Ellett, 1966, 1967; Woodhead 1978, 1979; Woodhead *et al.*, 1983). This work has been based upon a comprehensive collection of histological sections and embedded tissues kindly given to us by Dr. Alex Comfort. The sample used in this survey comprised 21 female and 12 male guppies ranging in age from 14 to 1907 days. The fish had been reared in laboratory aquaria under controlled conditions of temperature, salinity, light, and nutrition, and were designated as "normal growers" (Comfort, 1964). Based upon Comfort's actuarial data and upon our initial histological examination we earlier adopted the age of two years at a time in the lifespan at which we could make a convenient but realistic distinction between young and old individuals.

In this paper we report the changes with age that we have observed in the stratum griseum periventriculare of the midbrain roof. This particular layer is the fifth and innermost stratum of the optic tectum (Figure 1 and Plate 1). Our interest in this midbrain correlative center was aroused during separate experiments, when we observed a preferential accumulation there of an alkylating agent, thought to be a brain carcinogen in mammals (Woodhead *et al.*, unpublished).

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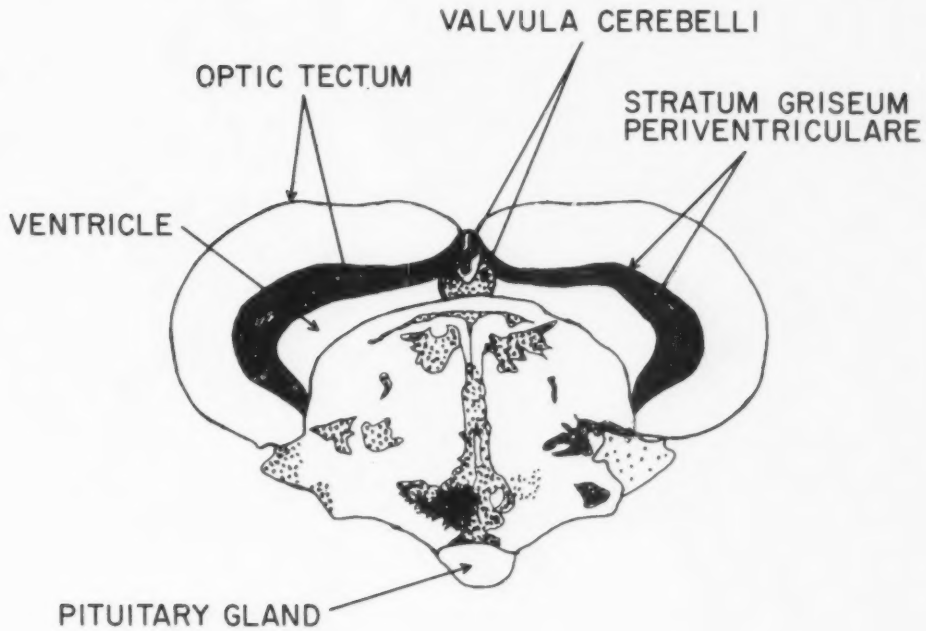
MATERIAL AND METHODS

Serial transverse sections were cut at 8 μm throughout the entire length of the body of the guppy *Lebistes reticulatus* and most of the sections were stained with Heidenhain's hematoxylin and eosin; sections from three females and two males were stained with Mallory's trichrome stain. Measurements were made of the stratum griseum periventriculare by projecting an image of a section onto paper of a standard thickness, and then tracing the outline of the brain and its component layers. These outlines were cut out and weighed in order to determine the proportion of the layer in relationship to the optic tectum and to the remainder of the brain tissue. The histological sections had been carefully prepared and evenly cut, and we were heedful to select sections that passed through similar areas of the midbrain—as far as possible we used sections that bisected the valvula cerebelli and the pituitary gland (Figure 1). Using the same section we also counted the numbers of cell layers in the stratum griseum periventriculare along four planes passing through the layer. In order to establish whether there are changes in specific areas of the brain, we first looked at the overall changes in brain length with age. An estimate was obtained of the length of the brain in relation to body length (measured from the tip of the snout to the end of the body cavity) by counting the number of sections throughout the body, and, since the thickness of the sections was uniform, we could obtain a value expressing brain length in relation to body length.

RESULTS

Changes in brain dimensions throughout the lifespan

One of the several controversies in the mammalian literature centers upon whether the usual histological techniques are sufficiently sensitive to reveal fine structural aging



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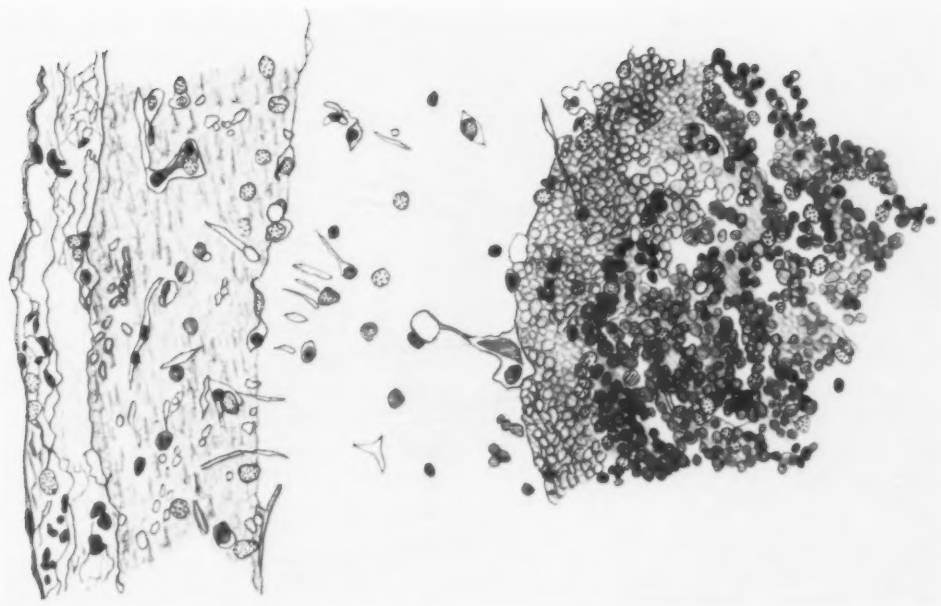

FIG. 1. Drawing of a transverse section through the midbrain of the guppy showing the stratum griseum periventriculare (top). Bottom is a drawing of the several layers of the optic tectum

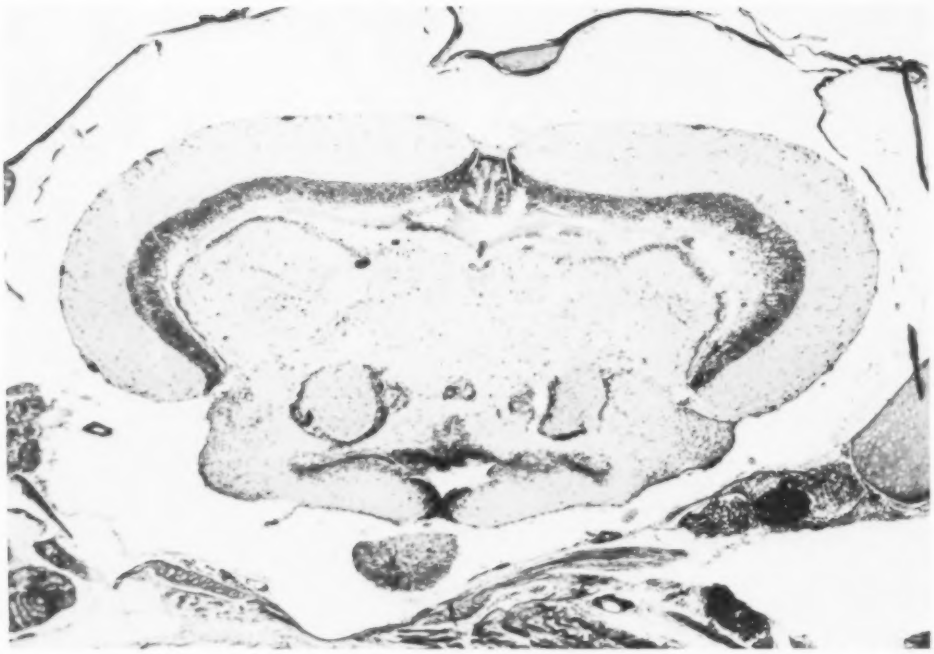

PLATE 1. Transverse section through the midbrain of the guppy showing the stratum griseum periventriculare. Magn. × 63.

changes in the brain; another revolves around the extent to which reported decreases and shrinkages in brain volume are artifacts due to improper fixation or compression of the sections (Sulkin 1961; Douglas *et al.*, 1981; McNeill, 1983). Our method of determining the ratio of brain length to body length by counting the number of sections included in the brain and the overall body overrides this difficulty.

Fish show a variety of growth patterns. Many species, including the female guppy, continue to grow throughout their lifespan although the rate of growth slows down considerably in older fish (Comfort, 1964). Other teleosts, such as the males of many small tropical species, numbering amongst which is the male guppy, show a pattern of growth quite similar to the mammalian type; that is, they exhibit rapid growth to a specific size after which growth ceases almost completely (Beverton and Holt, 1959; Comfort, 1964; Woodhead, 1978). Studies of growth and brain dimensions in herring (*Clupea harengus*) and in trout (*Salmo trutta*) have established that the growth pattern of the brain closely reflects that of the body so that, after larval and metamorphic stages, brain size maintains a constant ratio to body size (Packard and Wainwright, 1978). Our counts showed that in adult guppies of both sexes this relationship appears to hold true up until the age of two years, brain tissue being present in 30 to 40 percent of the body sections (Figure 2). In old males which had reached their specific size this proportionality was maintained. In older females, however, brain length was smaller in relation to body length, at a value of 20 to 30 percent. Females of two years of age have achieved about 75 percent of their eventual limiting size, and the growth curve at this point in their lifespan flattens out markedly

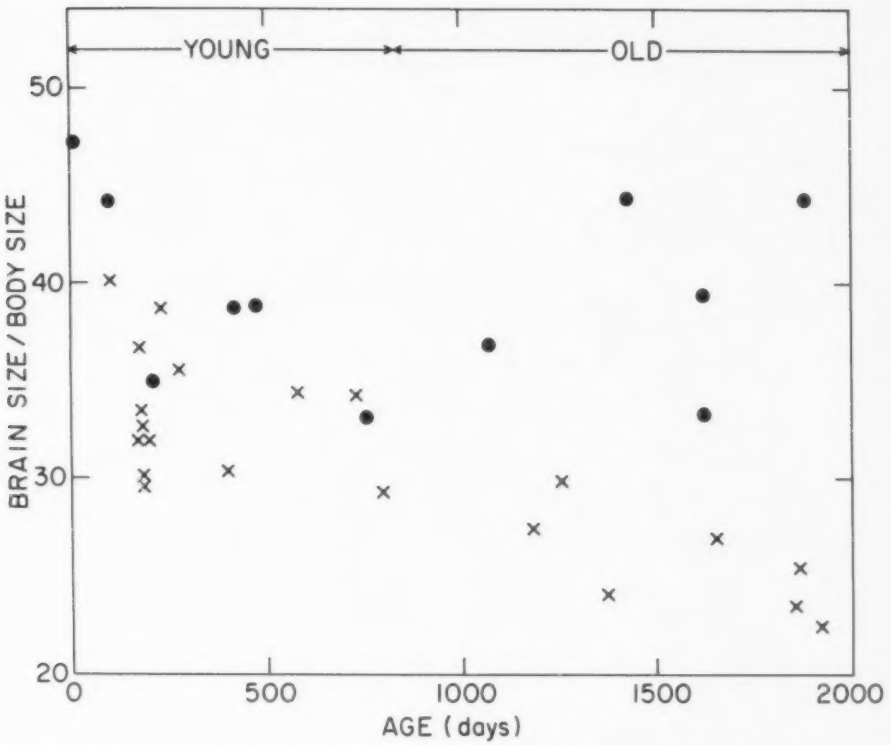

FIG. 2. The relationship between brain and body length in male (●) and female (X) guppies of various ages.

(Comfort, 1964). We believe that the decrease in relative brain size in old females does not reflect on overall loss of brain tissue in old age, since the number of sections upon which brain tissue was present remained steady. The decline can be almost entirely accounted for if one considers that the brain ceased to grow at the 75 percent asymptote when rapid body growth is over, whilst body growth continued at slow rate for the remainder of the lifespan (Figure 2).

Aging changes in the stratum griseum periventriculare

Had there been an overall loss of neural tissue in older guppies, it would have been difficult to distinguish whether any decrease in the size of the stratum griseum periventriculare was a real loss, or was a part and parcel of the shrinkage or cessation of growth of the organ as a whole. The measurements of brain size and the size of the optic tectum (from the transverse section), and of brain length enabled us to make this distinction quite clearly, especially in male fish.

Our measurements showed that the stratum griseum periventriculare was smaller in old fish than in young individuals of both sexes (Table 1). Cell counts also confirm that there had been a loss of cell layers in this stratum, from an average of 16 in young fish to about 11 in guppies over two years old (Table 2). We could not distinguish in these conventional light-microscope views whether this reduction reflected a loss of neurones, of glia cells or

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TABLE 1. STRATUM GRISEUM PERIVENTRICULARE IN YOUNG AND OLD GUPPIES EXPRESSED AS A PERCENTAGE OF (a) WHOLE BRAIN SECTION (b) OPTIC TECTUM.

	*Young**	*Old**	P *Value***
(a) Females	11.11 (11)	8.85 (5)	.006**
Males	10.10 (4)	7.35 (5)	.004**
(b) Females	22.95 (11)	20.99 (5)	.136
Males	23.05 (4)	19.73 (5)	.039**

*Number of fish in each group is given in parentheses.
**P is significantly different.

TABLE 2. NUMBER OF CELL LAYERS IN THE STRATUM GRISEUM PERIVENTRICULARE. THE NUMBER OF GUPPIES IN EACH GROUP IS GIVEN IN PARENTHESES.

	Young	*Old*	P *Value**
Females	16.4 (11)	10.9 (6)	.0003
Males	16.1 (5)	11.4 (5)	.0003

*P value significantly different in both sexes.

of other nervous system elements. Such differentiation can only be achieved by the use of electron microscopy (Kruger and Maxwell, 1966). The cell layers were closely packed together in young guppies but in older animals the cells had become loosely spread, and there were prominent acellular areas containing only a pink-staining exudate.

DISCUSSION

We have observed a loss of nervous tissue from the stratum griseum periventriculare of old guppies, in the order of 20 to 27 percent of tissue. The decrease in the size of the layer occurred at about the time in the lifespan that we had previously designated as the dividing point between a young and an old fish. The loss appears to be specific to this layer, and it is not a reflection of the shrinkage of the brain as a whole in old age; the changes do not appear to be an artifact of fixation nor of postmortem changes. Whether similar changes are happening in other strata of the optic tectum or other specific areas of the brain was not investigated.

The midbrain roof in fishes is the correlative center for optic and nonoptic exteroceptive sensations, the latter from the lateral line and vestibular sense organs. It is a major brain center concerned with maintaining the spatial orientation of the fish in relation to its external environment. Kirsche (1967) showed in crucian carp that there was a continuous generation of neurones by the matrix layers of the optic tectum so maintaining its integrity. How far a loss of cells in the optic tectum in old age, or failure to renew neurones, may contribute to functional behavioral deficits (if indeed there are any) is unknown. However, a loss of spatial orientation ability and atypical movements in older fish might render them more susceptible to death by predation.

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THE DEVELOPMENT OF MAMMOTROPH ADENOMAS IN PITUITARIES OF AGING FEMALE C57BL/6J MICE

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Abstract—The development of pituitary adenomas in female C57BL/6J mice was studied over the lifespan. Mammotrophs and somatotrophs, identified by immunocytochemical staining, appeared normal in 3–4 month old animals, with the exception of an occasional hypertrophic mammotroph and, in one gland, a nest of such cells. At 15 months, hypertrophic mammotrophs, somatotrophs and unstained cells occurred singly or in areas of hyperplasia; a mammotrophic adenoma was present in one gland. At 22 months, pituitaries contained mammotroph adenomas or adenoma-like mammotroph clusters within hypertrophic, hyperplastic areas as seen in 15 month old glands. Pituitaries from all 28–30 month old mice contained mammotroph adenomas; nests of hypertrophic cells were present in 5 of 8 glands studied. These results suggest that mammotrophic adenomas may arise from hypertrophic mammotrophs, which are often found in nests with other hypertrophic pituitary cell types. However, this study does not exclude the possibility that mammotroph adenomas and hypertrophic mammotrophs occur independently.

INTRODUCTION

SPONTANEOUS ADENOMAS of the anterior pituitary commonly occur during reproductive senescence in many laboratory rodents (Duchen and Schurr, 1976). In female C57BL/6J mice (Felicio *et al.*, 1980), pituitary adenomas are rare before 15 months, but are found in most mice by 24 months. Such tumors have been characterized by conventional histological staining methods, electron microscopy, and more recently, immunocytochemical techniques (Ito *et al.*, 1972b; Kovacs *et al.*, 1977; Schechter *et al.*, 1981; Tiboldi *et al.*, 1967; also, see Kovacs *et al.*, 1977b for a review). The majority of cells in the tumors appear to be mammotrophs and somatotrophs, although some autonomous tumor strains

contain cells which produce both prolactin (Prl) and growth hormone (GH) (Furth *et al.*, 1973; Ito *et al.*, 1972a; Ueda *et al.*, 1973). In a recent report, we identified the mammotroph in the pituitary tumors of old C57BL/6J mice by structural characteristics (Schechter *et al.*, 1981). Grossly elevated plasma prolactin (Prl), to > 500 ng/ml) is common in old rats with pituitary tumors (Huang *et al.*, 1976). In contrast, plasma Prl of old C57BL/6J mice with gross tumors tends to remain low (< 100 ng/ml) and is elevated to > 150 ng/ml only in a subgroup of those mice with large tumors (Nelson *et al.*, 1980). However, in view of the absence of the general hyperprolactinemia of old tumor-bearing mice and the degranulated appearance of many mice pituitary cells (Schechter *et al.*, 1981), immunocytochemical characterization with specific antisera was required to establish tumor cell type as mammotrophic.

The structural and immunocytochemical characteristics of mammotrophs were evaluated throughout the 30-month average lifespan of C57BL/6J mice. By 15 months, ovarian cyclicity is lost and is succeeded by a polyfollicular anovulatory condition during which plasma estradiol is sustained at modest levels while progesterone is very low (Nelson *et al.*, 1981). During the polyfollicular phase, the incidence of grossly observable tumors increases many-fold, from < 10% at 14 months to > 40% by 24 months (Felicio *et al.*, 1980). After 24 months, plasma estradiol drops to castrate levels in association with ovarian depletion (Gosden *et al.*, 1983), whereas plasma LH becomes elevated to castrate levels (Gee *et al.*, 1983). The hypertrophied gonadotrophs seen in this age group (Schechter *et al.*, 1981) are consistent with the elevated LH seen in other samplings (Gee *et al.*, 1983). Between 24 and 30 months, the incidence of tumors frequently is > 60% (Felico *et al.*, 1980). Importantly, LH was not elevated if pituitary tumors were present (Gee *et al.*, 1983). Thus, C57BL/6J mice display a striking model of menopause in humans involving similar decreases in plasma estradiol and elevations of LH.

Because mammotrophic tumors can be induced by chronic estrogen treatment in young rats (Clifton and Meyer, 1956; Sonnenschein *et al.*, 1974) and in young mice (Giok 1961), the steady but modest plasma estradiol experienced during the polyfollicular status just after the loss of cyclicity by 15 months could be a factor in tumorigenesis. It was therefore of interest to characterize mammotroph morphology at these major phases of reproductive senescence, to acquire further background for the construction of hypotheses on the origins of mammotrophic tumors. Additionally, we were able to evaluate if Prl and GH occurred in the same pituitary tumor cells, because both Prl and GH were reported in some autonomous tumor lines (Furth *et al.*, 1973); Ito *et al.*, 1972a; Ueda *et al.*, 1973), and because of evidence that 3-week treatments with estradiol can transform somatotrophs into mammotrophs in young rats (Stratmann *et al.*, 1974).

MATERIALS AND METHODS

Pituitaries from four age groups of female C57BL/6J mice were studied: 3–4 months (n = 7), 15 months (n = 7), 22 months (n = 6) and 28–30 months (n = 8). Mice were obtained from the Jackson Laboratory (Bar Harbor, ME) as virgins (3 months) or retired breeders (8 months) and maintained as previously described (Holinka and Finch, 1977). Glands were obtained by two methods: 1) After decapitation, the pituitaries were exposed and flooded with Bouin's solution or 4% neutral buffered paraformaldehyde in situ; or, 2) mice were anesthetized with sodium pentobarbital and perfused through the left cardiac ventricle with saline followed by 4% paraformaldehyde. Pituitaries were then removed, fixed, dehydrated by alcohol, embedded in paraffin, sectioned at 6μm on a rotary microtome, and mounted on gelatin coated slides. Immunocytochemical staining and absorption controls were performed using mouse Prl, mouse GH, rabbit anti-mouse Prl and monkey anti-mouse GH (Sinha *et al.*, 1972). Tissue sections were stained using the unlabelled antibody-enzyme methods (Sternberger 1970). Sections were incubated for 24 hours with anti-Prl or anti-GH at dilutions of 1:1400 and 1:1000, respec-

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TABLE 1. THE PRESENCE OF ABNORMAL CELLS IN THE PITUITARIES OF FEMALE C57BL/6J MICE AS A FUNCTION OF AGE.

Age	*# of Glands with Scattered Single Hypertrophic Mammotrophs*	*# of Glands with Areas of Hypertrophy and Hyperplasia (multiple cell types)*	*# of Glands with Mammotroph Adenomas*	*n*
3–4 months	4	1	0	7
15 months	2	5	1	7
22 months	0	5	6	6
28–30 months	4	5	8	8

tively. Adjacent sections were incubated with anti-Prl that was preabsorbed with Prl or GH anti-GH that was preabsorbed with Prl, or anti-rabbit gamma globin (AARGO 1:50, and peroxidase-anti-peroxidase (PAP), 1:200. Finally, sections were exposed to 3,3′-diaminobenzidine, 30 mg%, in 0.01 M Tris buffer pH 7.4, containing 0.03% H_2O_2, then dehydrated and mounted with coverslips.

RESULTS

Abnormal cell types as a function of age (described below) are summarized in Table 1.

3-month old mice

Most mammotrophs and somatotrophs at 3 months had normal morphology. The mammotrophs were irregular in shape and demonstrated the pockets of stained cytoplasm characteristic of this cell type (Figure 1). The somatotrophs were round or ovoid, with

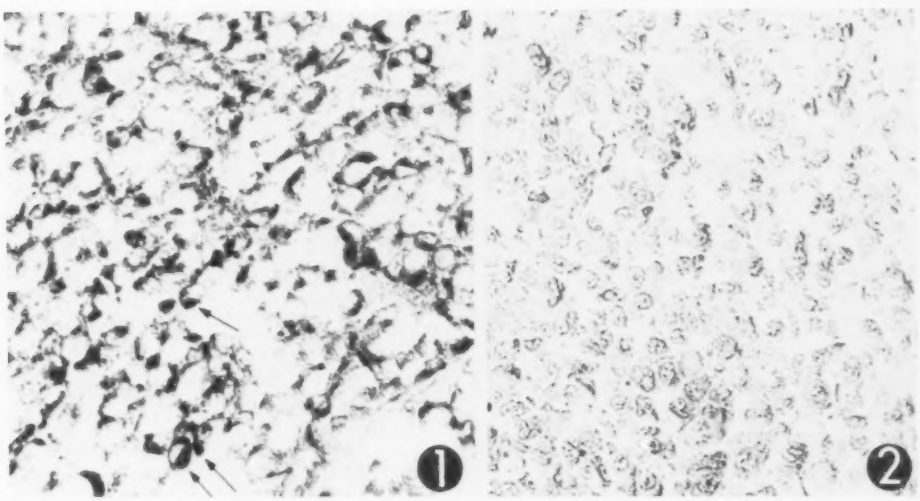

FIG. 1. Normal 3-month-old pituitary stained for Prl, showing pockets of stained cytoplasm typical of mammotrophs (arrows). 6 μ section. ($\times$ 450).

FIG. 2. Section near to that in Figure 1, stained with anti-Prl that was pre-absorbed with Prl. Although some background staining was present, no specific cytoplasmic staining was observed. 6 μ section. ($\times$ 450).

cytoplasmic staining which was more uniformly distributed about the nuclei than that of the mammotrophs. Single hypertrophic mammotrophs and somatotrophs were seen occasionally at this age, and one pituitary contained a nest of hypertrophic mammotrophs similar to those seen at later ages. Preabsorption of the primary antisera with their respective antigens eliminated specific cytoplasmic staining, although some background staining was present when anti-Prl was preabsorbed with Prl (Figure 2). Cross-absorptions, (e.g., anti-Prl preabsorbed with GH and anti-GH preabsorbed with Prl) gave the same results as for sections stained with untreated primary antisera. When sections stained for Prl were compared with adjacent sections stained for GH, no evidence was seen of any cells containing both hormones. The above results extend to the other age groups and will not be repeated below.

15-month old mice

Single hypertrophic mammotrophs and somatotrophs occasionally were present among normal-appearing cells, but the outstanding feature of the pituitaries at 15 months was the

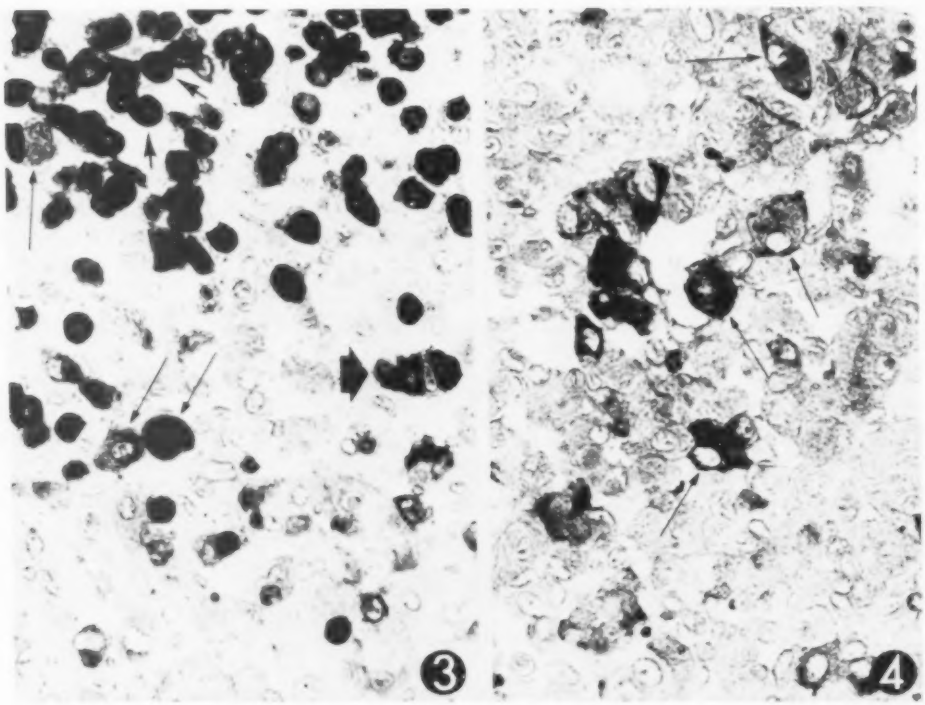

FIG. 3. 15-month old pituitary stained with anti-GH, showing a hyperplastic lesion containing hypertrophic somatotrophs (small arrows), somatotrophs in an abnormal configuration (large arrow), and many unstained cells. Some normal appearing somatotrophs are present at the periphery of the lesion (medium arrows). 6 u section. (× 450).

FIG. 4. Section near to that in Figure 3, showing hyperplastic lesion stained for Prl. Hyperplastic mammotrophs are present (arrows; compare with normal lactotrophs in Figure 1 at the same magnification) with numerous unstained cells. Some disruption and enlargement of vascular sinuses is apparent, with colloidal material remaining in some of the spaces (asterisks). This section was counterstained with methylene blue because the immunocytochemical background staining was insufficient for observation of unstained cells. 6 u section. (× 450).

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presence of areas of hyperplasia in most (5/7) glands. These hyperplastic areas contained mammotrophs and somatotrophs that usually were hypertrophic or in abnormal configurations (Figures 3 and 4), as well as large numbers of unstained cells. One pituitary also contained a small adenoma resembling the large tumors seen in the older age groups, as described below. Vascular lakes were present, usually in the form of thin slits penetrating between rows of tumor cells; some larger lakes were observed.

22-month old mice

Pituitaries at 22 months had characteristic areas of hypertrophy and hyperplasia containing mammotrophs, somatotrophs and unstained cells as seen at 15 months. However, these hyperplastic areas often contained nests of mammotrophs which, although not clearly hypertrophic, differed from normal mammotrophs in shape and distribution of stained cytoplasm (Figure 5). These cells resembled in size and shape those found in frank adenomas (Figure 6) in this and other age groups. The adenomas contained cords and clusters of cells, nearly all of which stained lightly or darkly with anti-Prl. Somatotrophs were rarely found within these adenomas. Vascular lakes were common (Figure 5) and sometimes contained hypertrophic or adenomatous mammotrophs.

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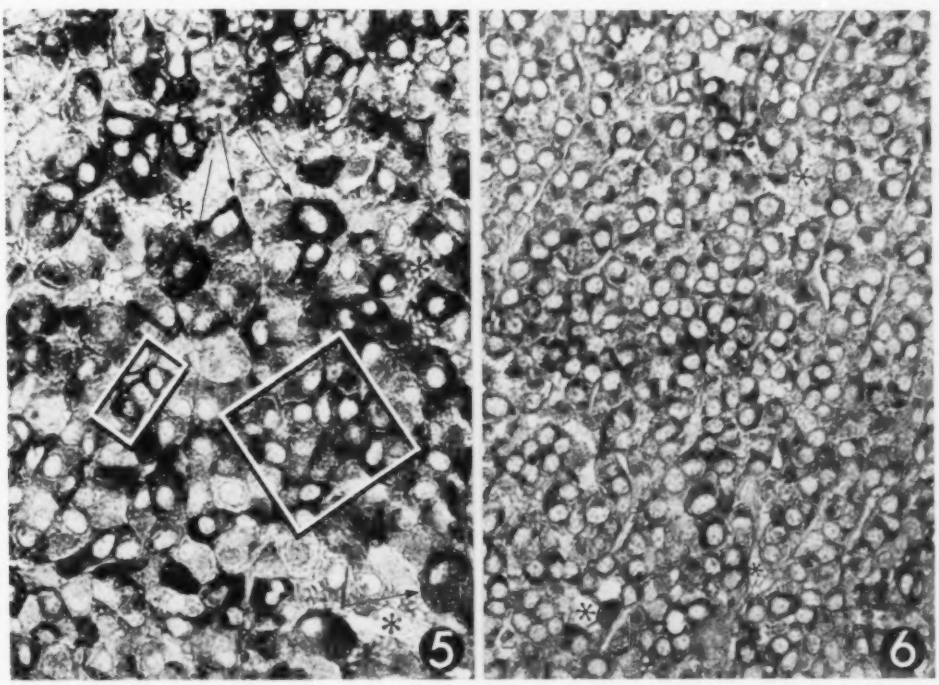

FIG. 5. Hyperplasia in a 22-month-old pituitary, stained for Prl. Hypertrophic mammotrophs are present (arrows) and nests of smaller mammotrophs (boxes) are similar in size and appearance to cells of mammotroph adenomas (see Figure 6). Asterisks denote vascular lakes filled with colloidal material.

FIG. 6. Adenomatous region from the same pituitary as in Figure 5, stained for Prl. The cells are arranged in cords and clusters typical of an adenoma, and virtually all are stained. The morphology of this tumor is similar to that of adenomas seen in other age groups. Small vascular lakes are indicated by asterisks, 6 μ section. ($\times$ 450).

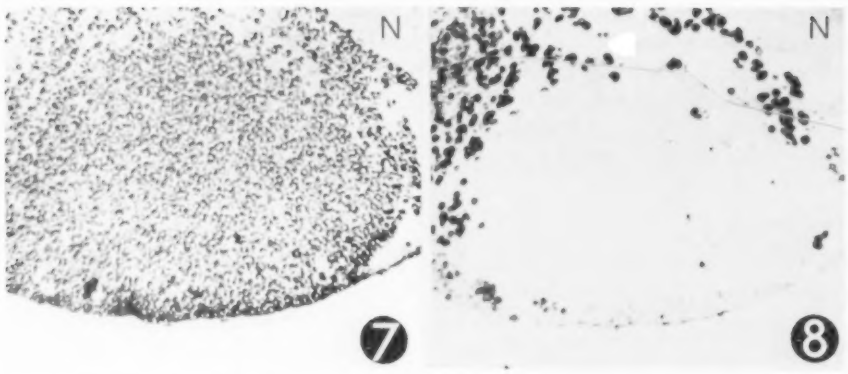

FIG. 7. Low power illustration of a mammotroph adenoma in a 28-month-old pituitary. The tumor is fairly well circumscribed, and some invasion of the neural lobe (N) has occurred. The tumor is riddled with small vascular lakes (arrows), which are poorly resolved at this low power. 6 μ section. ($\times$ 93).
FIG. 8. Photomontage of a section adjacent to Figure 7, stained for GH. Few somatotrophs are present within the body of the tumor (arrows), although they are numerous at its periphery. The neural lobe is demarcated by N. 6 μ section ($\times$ 93).

28–30-month old mice

Mammotroph adenomas similar to those in the 22-month age group were present in 100% of the oldest mice. These tumors often invaded the neural lobe (Figure 7); several expanded beyond the sella turcica, to indent the hypothalamus as observed during removal of the pituitary from the skull. Such tumors were encapsulated and were easily separated from adjacent neural tissue. Again, somatotrophs within these tumors were rare (Figure 8). Tumors were riddled with vascular lakes, ranging from slit-like spaces to large, colloid-filled areas loosely lined with Prl-containing cells. By gross observation of these and younger animals, there were no secondary tumor loci in the CNS or the periphery.

DISCUSSION

The pituitary adenomas which arise spontaneously during aging in female C57BL/6J mice consist mainly of mammotrophs, as established by immunocytochemical procedures with antibodies to mouse Prl. These results thus establish the previous hormonal assignment of these tumors in C57BL/6J mice, which was based on ultrastructural and tinctorial properties (Schechter *et al.*, 1981).

No evidence suggested that GH and Prl occurred concomitantly within the same cell; absorption controls indicated that crossreactive staining did not occur between the two hormones. However, some studies have demonstrated pituitary adenoma cells which produce more than one hormone (Furth *et al.*, 1973; Ito *et al.*, 1972; Ueda *et al.*, 1973). Interestingly, multihormonal cells usually were observed in induced or transplanted tumors, rather than in spontaneous tumors or normal pituitaries; however, Ueda *et al.* (1973) reported the existence of rare multihormonal cells in the latter. Although artifacts from crossreactivity of the antisera might be a factor in the findings of other investigators, it remains possible that pituitary tumor cells could produce both hormones.

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Numerous unstained cells were present in the hypertrophic, hyperplastic lesions. All or some of these cells may be gonadotrophic, somatotrophic or mammotrophic; the absence of immunocytochemical staining was perhaps due to previous hormone release or to hormone synthesis in quantities too small to allow immunocytochemical observation (Schechter *et al.*, 1981). In any case, the occurrence of hypertrophy and hyperplasia of somatotrophs and mammotrophs, as well as hypertrophy of gonadotrophs (Schechter, unpublished), appear to precede the development of frank mammotroph adenomas.

These data, considered with observations of adenoma-like clusters of mammotrophs within areas of hypertrophy and hyperplasia, suggest that mammotroph adenomas may arise from hypertrophic mammotrophs. Although no somatotrophic adenomas were observed here, some GH-containing tumors were found in another immunocytochemical study of aged mouse pituitaries (Schechter, Felicio, Nelson and Finch, unpublished). No gross gonadotrophic tumors were detected in C57BL/6J mice. It is intriguing that the adenomas contained few somatotrophs and no other hyperplastic cell types were observed (such cells would have been negatively stained in this study). Thus, the stimuli causing hyperplasia of several cell types may be distinct from those causing subsequent adenoma development. It remains possible that mammotroph adenomas arise independently of hypertrophic mammotrophs.

No secondary tumors were detected by gross observation. The presence of vascular lakes within tumor zones is an indication of capillary disruption (Schechter *et al.*, 1981). Although some tumors in the older animals extended beyond the sella between the brain and skull, these clearly were encapsulated with no evidence for metastasis. In general, spontaneous pituitary tumors are not metastatic (Berry and Caplan, 1979).

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CHANGES IN MOTOR ACTIVITY WITH AGE AND THE EFFECTS OF PHARMACOLOGIC TREATMENT

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Abstract—In a previous study the decline in the motor performance of old rats was determined to be differential. In this study, whether, and to what extent, this decline can be pharmacologically influenced was tested. Therefore, 27 month old rats were orally treated with several nootropics and d-amphetamine for six weeks. Food and water intake were determined. The rats were tested on spontaneous activity and on the tilting plane, climbing, and rotarod tests. The results showed that the nootropics only effected pronounced improvements on complex motor tasks such as on the rotarod test. In contrast, amphetamine treatment caused rather negative effects. This could be observed in the motor performance as well as in the food and water intake. Young rats did not have this sort of reaction to amphetamine. The nootropics were all well tolerated. Additionally, it was obvious that the untreated rats also showed a slight improvement in motor performance due to repeated practice.

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INTRODUCTION

NUMEROUS STUDIES point to a gradual and asynchronous impairment of the regulated systems in old age. (Finch, 1976; Frolkis, 1968; Hahn, 1979; Verzar, 1963; Wallace *et al.*, 1980; Keidel, 1975; Potvin *et al.*, 1980). In consequence a decrease in adaptation to endogenous and exogenous stimuli results. The appearance of the third phase of life occurs as a continuous process and claiming an organism to be "old" must be imprecise. One, among other meaningful delimiters, could be the 50 percent survival age of a given population (Burek, 1978; Rockstein *et al.*, 1977).

Motor performance is an example of a complex regulated system and could serve as a model for age-related changes and their possible pharmacologic improvement. In an earlier multi-task study designed to investigate motor ability, a performance profile was established in a cross-sectional study on a population of female wistar rats with a 50 percent survival age of 27 months (Jänicke *et al.*, 1983). The decline in performance was more pronounced the more complex the task was. No difference was observed between young and old rats for spontaneous activity and swimming. However a gradual reduction in abilities was noticed for maintaining balance on a gently tilted plane, being able to hang onto a horizontal wire for a maximum of one minute, for ascending a ladder and for being able to walk on a rotating rod (Jänicke *et al.*, 1983).

Since histological examination of hip and knee joints and the skeletal muscle of 34 and

6 month old rats revealed no pronounced differences (Jänicke *et al.*, unpublished), it is assumed that the decline in motor functions is due to deteriorated central control of peripheral mechanisms.

Other than a few non-comparable publications concerning stimulants (Bättig, 1961; 1963; Plotnikoff *et al.*, 1977), there are few studies in which old rats have been given medication with the goal of improving their motor performance. In the present study the extent to which decline in motor ability can be pharmacologically effected was tested. The drugs used were d-amphetamine and the nootropics pyrithioxin, piracetam, hydergin®, centrophenoxine and Ro-13-5057.

Which of the pharmacodynamic characteristics have therapeutic relevance is hardly known. Generally, one can say that the application of these agents obviously effected a higher functional status and thus lead to a general improvement in alertness which favored the motor performance. The increase of sensomotoric coordination, intensity and duration of attention could be understood in terms of vigilance and its improvement (Diamond and Lazarus, 1974).

MATERIALS AND METHODS

Female wistar rats of two age groups (6 and 27 months old) were used in the experiments (breeder: Hagemann, Bösingfeld/Lippe). The animals were housed two per cage (39 × 23 × 15 cm). Body weight, food intake (ad libitum) and the amount of the substance consumed were determined daily.

For each substance 20 old rats and a total of 40 controls were used. In addition, 20 young animals each, received either d-amphetamine or water. The substances were administered orally through the drinking water. After determining the daily amount of consumption, the animals were offered enough fluid to ascertain that they had ingested the desired dosis: piracetam – approximately 250 mg/kg/day, pyrithioxin – approximately 50 mg/kg/day, hydergin® – approximately 0.5 mg/kg/day, centrophenoxine – 100 mg/kg/day, Ro 13-5057 – 30 mg/kg/day, d-amphetamine – 2 mg/kg/day.

Spontaneous activity

Spontaneous activity of individually housed rats, that had adapted to the test situation, was measured by a motilimeter. Each animal was equipped with a motilimeter which works under the principle of change in capacity and continuously registers the spontaneous activity of the individual rat in its cage. Thus, all motility greater than, for example, chewing movements were recorded. Measurements were made during the dark phase (7:00 p.m. to 7:00 a.m.). We tested six animals per group.

Motor tests

1) Tilting Plane Test. Analogous to the methods of Arvola *et al.* (1958), the rats were placed on a horizontal plane surrounded by 6 cm. high walls. Within 3–4 seconds the plane was tilted from 0° to 90° and the angle at which the animal could no longer prevent sliding was determined. This task demands an especially quick reaction time and vestibular information processing. The arithmetic mean of three test trials was calculated.

2) Horizontal Wire Test. Rats were allowed to grasp a metal rod with their forepaws (0.4 cm diameter, 83 cm long) and were then released. The wire was horizontally fastened 40 cm above a table top in order to allow enough space and time for falling reflexes. The duration of hanging, using two or four extremities and/or the tail, was measured. Trials were ended after 60 seconds and animals were given five minute rests between trials. The longest hanging time out of three trials was evaluated.

3) Climbing Test. Rats had to climb up a 60 cm long, 7 cm wide metal ladder, with rungs spaced 4 cm apart. The motivation for climbing consisted of reaching a goal box at the top of the ladder, and of avoiding falling into a wood-chip lined cage below. After two or three practice sessions, each animal was tested three times, with a five-minute rest between each trial. Measurement of the number of grasps with the forepaws and the climbing time allowed determination of the stepping speed and the stepping distance. The arithmetic mean of the three trials was calculated.

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4) Rotarod Test. Rats were placed on a rotating rod and had to walk in the direction opposite that of rotation. The rod (5 cm diameter, 20 cm long) was fastened 30 cm above a table top. A motor provided the three rotation speeds of 10, 20 and 40 rpm. On the first day, rats had to spend two minutes on the slowly rotating rod (10 rpm). On the two successive days, the speed of the previous day was doubled (day two – 20 rpm, day three – 40 rpm). To meet the performance criterion 60 percent of a group had to remain on the rotarod for two minutes. The parameters were each animal's frequency of starts and the amount of time spent on the rod before falling off. The animals were given five-minute rests between starts and the number of starts was limited to 15/day. Group performance was expressed as a median. For statistical analyses of the data the Kolmogrov-Smirnov Test was applied to the differences.

Procedure

One month prior to administration of the substances, experimental and control animals were exposed to the motor tasks once in order to prevent shifts in performance caused by novelty of the new tasks. The motor tasks were performed after three and six weeks of treatment with the substances.

RESULTS

Body weight, nourishment

No noticable influence on food and fluid intake, or body weight was observed after the rats had been treated for six weeks with pyrithioxin, piracetam, hydergin®, centrophenoxine or Ro-13-5057. However, there were clear effects of d-amphetamine treatment *in old rats* (Figure 1). For most of the time fluid intake was reduced by 40 percent, increasing substantially only after d-amphetamine treatment was stopped. The anorexia (50 percent reduction in food intake during the first few days) was soon normalized. Body weight dropped an average of 45 g until the 15th day of treatment. After drug treatment was stopped and in spite of normal food and water intake, an approximation to the body weight of control animals never occurred. In contrast, young rats treated with d-amphetamine did not reduce food intake and only slightly reduced fluid intake. The body weight was parallel to that of controls and increased an average of 10 g after cessation of treatment.

Spontaneous activity

The night-phase spontaneous activity of old rats did not increase as compared to control animals neither after ingesting nootropics nor after taking d-amphetamine. After cessation of d-amphetamine treatment the spontaneous activity level was drastically reduced (50%). This was not the case for the other drugs.

Motor tests

All drugs tested had a relatively uniform effect on the performance level of the rats. No improved results were observed for the tilting plane and the horizontal wire tests. After six weeks, there was a slight improvement on the climbing test.

In contrast, improvements were noticed on the rotarod test, which requires rather complex coordination. No pronounced effect was noticed after three weeks of application. However, after six weeks of treatment with pyrithioxin, centrophenoxine, and Ro-13-5057, the animals improved their performance by reducing the start frequency by 50 to 60 percent as compared to control animals at the 20 rpm speed (Figure 2); for piracetam and hydergin® the improvement was 25 to 40 percent. Amphetamine, however, induced a poorer performance on the rotarod test. The running time per start was decreased, implicitly increasing the number of starts.

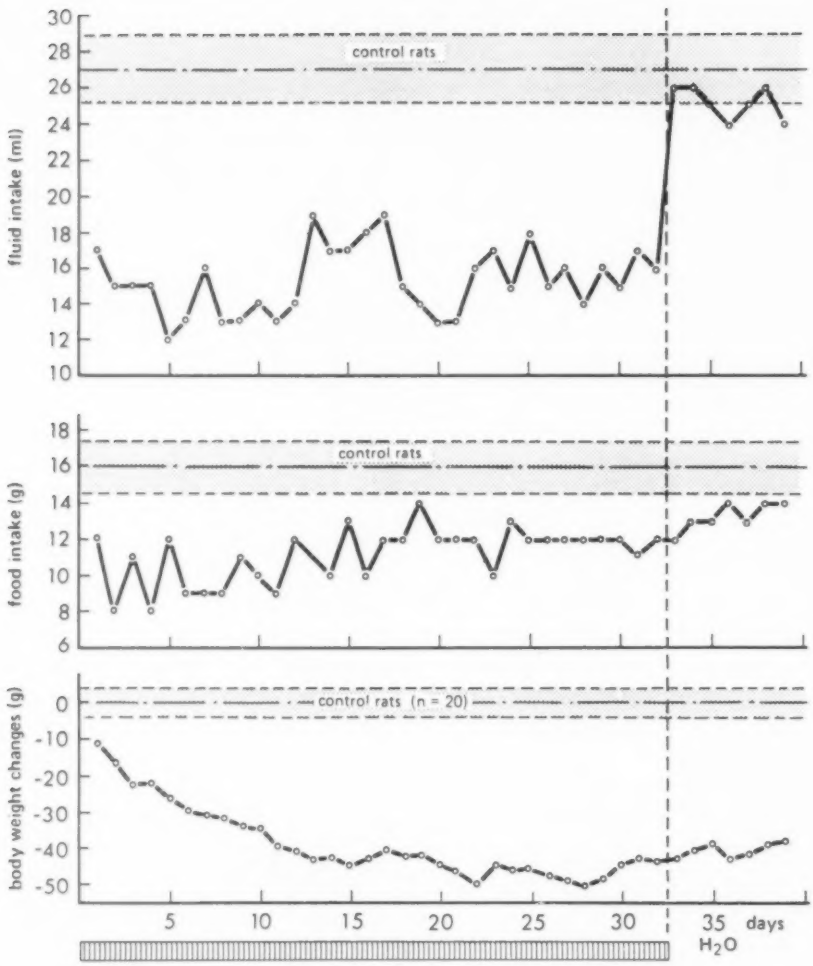

FIG. 1. Effect of d-amphetamine (2 mg/kg/day) and withdrawal on body weight, food and fluid intake of 27 month old rats compared to controls, $\bar{x}$, n = 20 each.

Both the low and the high rotation speeds turned out to be unsuitable for demonstrating a pharmacologic effect of the substances tested. Ten rpm was most likely not challenging enough since a large percentage of the control animals had no problems meeting the criterion. In contrast 40 rpm obviously was too difficult in that it could not be mastered either through practise or through drug administration. At this speed the limit at which the rats were capable of adequately reacting was probably exceeded and a decompensation followed. This corresponds to the studies by Steinhagen-Thiessen *et al.* (1980) and Edington *et al.* (1972) who showed a limit in the performance improvement of old animals due to training effects.

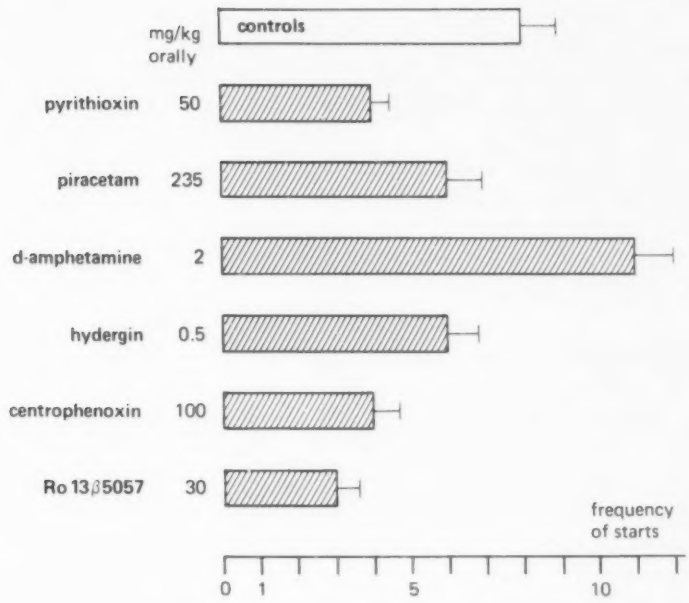

FIG. 2. Number of starts on rotarod test (20 rpm) for 28 month old rats after chronic treatment (6 weeks) with different substances as compared to control values; Median +/− SEM, n = 20 per substance ($p < 0.025$).

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DISCUSSION

Body weight, nourishment

Spontaneous activity

Out of all the substances administered, only amphetamine effected the food and water intake and body weight of the animals. This is in agreement with the literature. It is reported that older animals show a more or less drastic weight loss after an amphetamine application of even short duration. Spengler and Waser (1959) determined a sharp weight loss in male rats (330–400 g) after one injection of 2.5 mg/kg i.p. of amphetamine. Ziem *et al.* (1970) observed a 10 percent reduction of food intake in 13 month old female rats during the first days of amphetamine treatment (10 mg/kg i.p.). In spite of continued treatment this effect however, normalized after ten days. For 26 to 30 month old rats, Farner (1961) reported a 20 percent decrease in food consumption (2 mg/kg i.p. amphetamine).

Spontaneous activity is a relatively stable function throughout life and is only slightly, but differentially influenced by the substances. Our results do not indicate a considerable change under nootropic and amphetamine administration. Thus, an elevated activity level during testing is unlikely. Farner and Verzar (1961) recorded lower values with respect to intensity and duration of amphetamine treatment. Other authors show similarly divergent

results in that they observed hyperactivity (Puri *et al.*, 1976, 0.51-1 mg/kg i.p.). Whether the discrepancies in the results are due to changed pharmacokinetics (Honecker and Coper 1975) to age-related insufficiencies in the catecholamine system, or are due to different methodical procedures remains undecided. There is, however, no doubt that the capacity for adaptation in old age is reduced for these functions as was apparent from the counter-reaction which followed the interruption of d-amphetamine treatment (Kennedy, 1957). The observed strong counter-reaction at this relatively low dose is, like the reaction to food and water intake, an indicator of increased insufficiency of the regulated system to adapt to interferences since this sort of reaction was not observed in the young animals.

Motor tests

From the results of the tilting plane, horizontal wire, and climbing tests, it became apparent that even old, untreated rats were able to improve their performances by training. Obviously the pre-test and test after three weeks of treatment brought about a certain familiarization with the task demands. They, however, did not achieve the performance level of young animals (Jänicke *et al.*, 1983). It is striking that the drugs tested primarily influenced the performance of complex movement patterns (rotarod test) as opposed to less demanding activities such as in the tilting plane test.

Remarkably amphetamine did not cause improved performance on any of the tests, but rather brought about a worse performance level. The observed weight loss could hardly have been the cause for the decreased performance confirming previous results (Jänicke *et al.*, unpublished). Additionally, there are some interesting reports about interferences with the structure and function of Purkinje cells in the cerebellum after chronic amphetamine application (2 mg/kg) which could explain the observed poorer performance (Freedman and Marwaha, 1980).

Generally, it is possible that the administration of amphetamine could compound the already existing age-related decline in adaptation abilities to a given stimulus making the processing of task-related stimuli more difficult. This could lead to a less differentiated reaction spectrum and curtailed performance profile (Hicks *et al.* 1980).

There are a few references for comparison concerning the effects of the other agents. The substances tested in this study are increasingly being used in pharmacologic therapy for are-related disorders. Some indications of positive influences on vigilance and improvements following brain injuries were observed in clinical research (Coper and Kanowski, 1983). These changes are reflected in EEG measurements for example for pyrithioxin (Künkel and Westphal, 1976).

It is stressed that a compensation is obviously needed only under the demands of a complex pattern of movement, since the controls as well as the treated rats clearly demonstrated sufficient flexibility in successfully solving less complex tests by means of practice and repetition. The maximum level of functional capacity of which an organism is capable needs to be described first, as done by Steinhagen-Thiessen *et al.* 1980, de Vries, 1970 and Edington *et al.*, 1972 in order to then aim for an eventual pharmacological intervention. This procedure allows the individual to use its behavioral and physiological repertoire to a wide extent. Some studies in old humans have shown that this individual variability (plasticity) is not reduced (Baltes and Willis, 1982; de Vries, 1970) as was previously believed.

SUMMARY

Pharmacologic influences on the decline in motor performance of old rats were studied. For over six weeks, 27 month old female rats were treated with one of the agents pyrithioxin (approx. 50 mg/kg/day) piracetam (approx 250 mg/kg/day), hydergin® (0.5 mg/kg/day), Ro-13-5057 (30 mg/kg/day), centrophenoxine (100 mg/kg/day), or with the psychostimulant d-amphetamine (2 mg/kg/day). In addition, the reaction of young rats was tested after the same d-amphetamine dosage. The motor performance of the rats was tested on different tasks of varying complexity: spontaneous activity, tilting plane, horizontal wire, climbing test, and rotarod tests. The results were compared with those of the animals before treatment and with a control group.

In contrast to tolerating the other substances well, the old animals reacted to amphetamine with a drastic reduction in food and fluid intake. The anorexia normalized after some time (Figure 1). Spontaneous activity was not significantly increased. The animals were, however, clearly sedated after drug administration was stopped. In comparison, the young animals showed only slight variations in body weight and no substantial change in spontaneous activity both during and after amphetamine administration.

The results of the motor tests show that the substances, except for d-amphetamine, brought about improvement on the rotarod test (Figure 2). D-amphetamine administration lead to striking decline in old rats especially on the rotarod test, while no change in performance was observed in young rats. No differences between treated rats and control animals were observed for the other test tasks. In addition, the importance of repeated practice of a movement pattern for a stable or improved performance in age was recognized.

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A STUDY OF DIETARY RESTRICTION AND LIFESPAN IN THE ROTIFER *ASPLANCHNA BRIGHTWELLI* MONITORED BY CHRONIC NEUTRAL RED EXPOSURE

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Abstract—The rotifer *Asplanchna brightwelli* could be exposed to concentrations of neutral red at 0.1 to 0.75 μg/ml for two consecutive generations with no adverse effect on the lifespan or fecundity of either generation. Chronic exposure to higher concentrations of neutral red caused reductions in both lifespan and fecundity of the rotifers. Because neutral red stains the *Paramecium* which serve as a food source for the rotifers, the extent of red coloration in each rotifer's gut gave an approximation of how much food that rotifer had consumed. The *Paramecium* concentration had to be reduced to 100 per ml before pale gut coloration provided clear evidence of reduced food intake or dietary restriction. The group of rotifers with reduced food intake had a significantly longer lifespan than any other group. Their lifespan was 14.2% longer than that of control rotifers.

INTRODUCTION

IT IS well established that dietary restriction increases the lifespan of the rotifer (Fanestil and Barrows, 1965; Verdone-Smith and Enesco, 1982) and of other invertebrates such as *Tokophyra lemmarum* (MacKeen and Mitchell, 1975). In these studies, dietary restriction was imposed either by lowering the food supply or by increasing the feeding intervals. The purpose of the present study was to find a direct means of verifying food intake by the rotifer to establish a more precise correlation between food consumption and lifespan. The approach adopted was to stain the *Paramecium* on which the rotifers feed with a vital dye which could be visualized in the gut of the rotifer. However, before such a study could be attempted, it was necessary to establish whether or not the vital dye would affect the lifespan of the rotifer. As a whole, vital stains are used only for a limited period of time to follow a given developmental stage or a series of cytological events. The stains are rarely maintained for any length of time.

The rotifer *Asplanchna brightwelli* is useful for aging studies because of its relatively short lifespan and ease of maintenance. It has been used for a variety of aging studies in this laboratory (Enesco and Holtzman, 1980; Verdone-Smith and Enesco, 1982a, b, c; Sawada and Enesco, 1984). In these studies, we recognized that our observations would have been facilitated if the rotifers could have been stained throughout their lifespan with a vital dye that would not alter their natural lifespan. The first series of studies presented

here was thus initiated to determine whether we could find a concentration of neutral red which would not alter either the lifespan or fecundity of the rotifer. The second series of experiments was designed to use the neutral red to assess the level of dietary restriction imposed upon the rotifers and to allow a more direct correlation between food consumption level and lifespan.

MATERIALS AND METHODS

Culture Methods

Cultures of the rotifer, *Asplanchna brightwelli* clone 4B61 (originally obtained from Dr. John Gilbert, Dartmouth College, Hanover, NH) were grown as indicated by Enesco and Verdone-Smith (1980) in a Cerephyll preparation with *Paramecium caudatum* and *E. coli* as food sources. There were 48 rotifers in each experimental group.

Neutral Red

A stock solution of 1 mg/ml neutral red chloride (lot No. 021387; C.I. No. 50040, Aldrich Chemical, Milwaukee, Wisconsin) was first prepared by dissolving it in distilled water. The neutral red was then added to the culture medium at concentrations of 0.1, 0.5, 0.75, 1.6 and 3.2 μg/ml.

New born rotifers were individually placed into each of the 24 wells of a 24-welled tissue culture dish (Costar No. 3524 Tissue Culture Chamber). Each well contained 2.5 ml of the culture medium with the appropriate concentration of neutral red. Lifespan and fecundity observations were made every 6 hours. To replenish the medium, 1.0 ml of old medium was removed and replaced with fresh medium at each observation period. The rotifers were incubated in the dark at 19 $\pm$ 0.5 °C and were exposed to light for a maximum of 20 minutes at each observation period so that lifespan and fecundity measurements could be made.

Dietary Restriction

Paramecia densities of 25, 50, 100, 200, 250, 500 and 1000 paramecia/ml were prepared from stock paramecia cultures by diluting the medium with appropriate volumes of the Cerephyll preparation which did not contain any paramecia. Neutral red was added to all the media at a concentration of 0.5 μg/ml. The paramecia concentration in each medium was verified by counting the number of paramecia in a 100 μl sample spread on a grid. Exactly 2 μl of 1000 μg/ml neutral red solution was added to each 100 μl samples. The final concentration of 20 μg/ml neutral red was high enough both to kill the paramecia and to stain them intensely, allowing quick counts to be made. The samples were placed on a 60 $\times$ 15 mm Petri dish with 2 mm grids (No. 3160: Costar, Cambridge, Mass.) and the number of paramecia was counted using a dissecting microscope. The paramecia food supply was thus progressively decreased as a means of imposing dietary restriction on the rotifers. The extent of gut coloration was evaluated as bright red or pink when the rotifers were 2 days old.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by a *post hoc* Tukey test were performed as indicated in Brunning and Kinz (1977) and Sokal and Rohlf (1981).

RESULTS

The neutral red supplied in the culture medium was readily taken up by the paramecia at all concentrations used. The paramecia accumulate the dye either by diffusion or by ingestion processes. However, at the lowest concentration used in this study (0.1 μg/ml) the rotifers themselves did not become sufficiently stained. The dye concentration had to be at least 0.5 μg/ml in order for the rotifers to be clearly visible. Microscopic examination showed that the gastric gland, the corneal ciliated area of the head, and the brain of the rotifers were often lightly colored by the neutral red. However, the most prominent stain was in the gut, due to the ingestion of stained paramecia. When the stained paramecia were assimilated, there was a subsequent loss of coloration in the gut of the rotifers. This process was complete within 12 hours after ingestion of the stained paramecia.

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Table 1 presents lifespan data for groups of 48 rotifers grown in several concentrations of neutral red as compared to untreated control rotifers. Five concentrations of neutral red were used, ranging from 0.1 to 3.2 μg/ml. Statistical analysis using the one-way ANOVA followed by the *post hoc* Tukey test showed that there was a significant decrease in the mean lifespan of the rotifers exposed to the highest concentration of neutral red: 3.2 μg/ml (alpha = 0.05). However, a one-way ANOVA showed that there were no significant differences in mean lifespan for the other groups of rotifers exposed to neutral red at concentrations of 0.1 to 1.6 μg/ml ([F 4, 235] = 0.746, $p > 0.5$). Thus, neutral red did not have any effect upon the lifespan of the rotifers at the four lowest concentrations used.

Data on the influence of various concentrations of neutral red on rotifer reproduction is also presented in Table 1. This data shows that neutral red had very little effect on fecundity at any concentration. Statistical analysis using the one-way ANOVA followed by the *post hoc* Tukey test showed that the reproductive period was significantly longer for the rotifers exposed to 1.6 μg/ml neutral red than for any other group (alpha = 0.01). However, the analysis showed that there were no significant differences in the number of offspring for any group as a result of neutral red exposure.

The offspring of the rotifers cultured in neutral red for their entire lifespan were in turn exposed to the same concentration of the strain to determine the effects of chronic exposure to neutral red for two generations. Table 2 shows the lifespan and fecundity data for second generation rotifers exposed to neutral red. The data show that rotifers exposed to 0.1, 0.5 and 0.75 μg/ml neutral red for two generations were normal with respect to lifespan, length of reproductive period and mean number of offspring. Statistical analysis confirmed these observations. Statistical analysis further showed that there were no differences in any parameter measured at the three lowest concentrations of neutral red used. Only at 1.6 and 3.2 μg/ml neutral red were lifespan and fecundity significantly reduced.

Having established that neutral red at low concentration would not in itself alter the lifespan or fecundity of the rotifer, we were now able to use it as a tool to verify the extent to which dietary restriction would alter lifespan. Dietary restriction was imposed by lowering the paramecia concentration of the medium. Since the paramecia were stained with neutral red, the extent to which the rotifers were ingesting the paramecia could be gauged

TABLE 1. MEAN LIFESPAN AND REPRODUCTIVE PROFILE FOR *A. BRIGHTWELLI* REARED WITH DIFFERENT CHRONIC NEUTRAL RED CONCENTRATIONS. (n = 48)

Neutral Red Concentration (μg/ml)	*Mean Lifespan (days ± S.E.)*	*Ave. Reprod. Time (days ± S.E.)*	*Ave. No. Offspring/Rotifer*
0.0 (Control)	5.38 ± 0.12	3.08 ± 0.12	9
0.1	5.14 ± 0.13	2.89 ± 0.12	8
0.5	5.30 ± 0.15	3.08 ± 0.16	9
0.75	5.27 ± 0.10	3.11 ± 0.13	9
1.6	5.46 ± 0.17	3.79 ± 0.15*	10
3.2	4.81 ± 0.10**	2.85 ± 0.12	8

*Significantly longer than control at alpha = 0.01
**Significantly shorter than control at alpha = 0.05

TABLE 2. MEAN LIFESPAN AND REPRODUCTIVE PROFILE FOR *A. BRIGHTWELLI* REARED FOR TWO GENERATIONS UNDER DIFFERENT CHRONIC NEUTRAL RED CONCENTRATIONS. (n = 48)

Neutral Red Concentration (μg/ml)	*Mean Lifespan* (days ± *S.E.*)	*Ave. Reprod. Time* (days ± *S.E.*)	*Ave. No. Offspring/Rotifer*
0.0 (Control)	5.42 ± 0.11	3.16 ± 0.15	9
0.1	5.36 ± 0.10	3.09 ± 0.14	9
0.5	5.54 ± 0.13	3.47 ± 0.11	8
0.75	5.33 ± 0.12	3.09 ± 0.11	9
1.6	4.58 ± 0.15**	2.75 ± 0.14**	7**
3.2	4.45 ± 0.11*	2.50 ± 0.15*	7**

*Significantly different from control at alpha = 0.01
**Significantly different from control at alpha = 0.05

by the degree of red coloration in the gut. Table 3 shows that there was no difference in the lifespan of rotifers provided with paramecia at densities between 200 and 1000 per ml. At all these densities the gut coloration was bright red, indicating a uniform level of food ingestion. The lifespan was longer only for rotifers fed with the lowest concentration of paramecia (100/ml): here the gut coloration was pink and considerably paler than the bright red observed in the other groups. The pale gut coloration confirmed that the rotifers were ingesting fewer paramecia.

When statistical analysis was applied to this data, the ANOVA test showed that there were significant differences in lifespan among the 5 groups tested ([F 4, 235] = 23.76, $p < 0.001$). Subsequent analysis using the *post hoc* Tukey test showed that the rotifers fed with the lowest concentration of paramecia (100/ml) had a significantly longer lifespan than any other group (alpha = 0.01). Table 3 further shows that the average number of offspring produced by the rotifers was not affected by any alterations in paramecia density.

It should be noted that paramecia densities of 25 and 50 paramecia/ml were prepared and supplied to groups of rotifers. These concentrations proved to be two low and resulted in early starvation of some of the rotifers. Therefore, these data are not included.

TABLE 3. THE RELATIONSHIP BETWEEN PARAMECIA DENSITY, BUT COLORATION AND LIFESPAN IN *A. BRIGHTWELLI* WITH NEUTRAL RED CONCENTRATION OF 0.5 μG/ML. (n = 48)

Paramecia Density (paramecia/ml)	*Mean Lifespan* ± *S.E.M.* (days)	*Gut Coloration After 2 Days*	*No. of Offspring*
100	6.19 ± 0.09*	pink	8
200	5.52 ± 0.10	bright red	8
250	5.59 ± 0.13	bright red	9
500	5.39 ± 0.08	bright red	8
1000 (control)	5.40 ± 0.12	bright red	9

*Significantly longer at alpha = 0.01

DISCUSSION

The results of the present study indicate that chronic exposure to neutral red at concentrations from 0.1 to 0.75 μg/ml for two generations had no effect on the lifespan or the reproductive profile of rotifers. This data establishes that 0.75 μg/ml is the highest concentration of neutral red that can be used for chronic administration to rotifers without altering their natural lifespan or reproductive patterns. At the other end of the scale, a concentration of 0.1 μg/ml neutral red is too dilute for clear staining of the rotifers. We conclude that the optimal concentration of neutral red which produces clear staining and unaltered physiology is between 0.5 to 0.75 μg/ml. Conn (1961) describes neutral red as a weakly basic, non-toxic vital dye of the azin group. This is the first report that we are aware of where an organism has been maintained in a vital dye throughout its lifespan, or for two successive generations.

Having established that neutral red would not in itself alter lifespan, we were able to use this dye to evaluate food intake as correlated with lifespan in dietary restriction experiments. The presence of food alone does not assure that an organism is eating the food source. When the rotifers ingest the neutral red stained paramecia, the degree of coloration in their gut gives an approximation of their food intake. Since the rotifers provided with paramecia at 200 to 1000 paramecia/ml had bright red coloration, we have no evidence of any dietary restriction taking place in these groups. The lifespan of these groups was no different from the lifespan of control rotifers. In contrast, the rotifers fed with 100 paramecia/ml showed evidence of reduced food intake as evidenced by the pale pink color in their gut. It is striking that this group where there was clear evidence of reduced food intake also had a significantly increased lifespan. This is the first report where actual food intake has been assessed in experiments dealing with dietary restriction in invertebrates.

Much discussion has surrounded the results obtained using different methods of dietary restriction in mammalian systems. These methods are described by Barrows and Kokkonen (1977); they include (1) reduction of daily food intake, (2) intermittent feeding, and (3) reduction of protein intake. While the third method is not feasible for the type of predatory rotifer used in the present study, it is interesting that we can compare the outcome of the first two methods of dietary restriction on *A. brightwelli,* and that the results are remarkably similar. The first method (reduction of food intake) was used in the present study. The lifespan of the restricted rotifers (6.19 days) was 14.2% longer than that of the control rotifers (5.42 days). The second method (intermittent feeding) was used in a previous study (Verdone-Smith and Enesco, 1982a). The lifespan of rotifers fed every 36 hours (6.08 days) was 11.8% longer than that of control rotifers (5.44 days). Thus intermittent feeding and reduction of food intake are equally effective in extending the lifespan of the rotifer. It is interesting to note that the results obtained with this short lived invertebrate are similar to those reported for mammalian systems.

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THE EFFECT OF LIGHT, DARK OR ALTERED CIRCADIAN CYCLE ON THE LIFESPAN OF THE ROTIFER *ASPLANCHNA BRIGHTWELLI*

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Abstract—Rotifers were maintained in various light conditions for at least 20 generations. In the first set of experiments lifespan and fecundity data were compared for groups of rotifers maintained under LL (continuous light), DD (continuous dark) or LD 12:12 (control, 12 hr. light 12 hr. dark). The mean lifespan of the rotifers cultured in DD conditions was significantly increased as compared to the LL or LD 12:12 groups but there were no fecundity differences. There was no alteration in lifespan or fecundity of the LL group as compared to the LD 12:12 control. In a second set of experiments, an LD 6:18 cycle was imposed to determine whether a shift in the circadian cycle would influence lifespan. Rotifers exposed to the LD 6:18 cycle and to the DD conditions showed an 18% and 22% increase in mean lifespan respectively. The results are interpreted to demonstrate that lifespan is influenced by the circadian cycle.

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INTRODUCTION

THE EXPERIMENTS reported here were designed to determine whether the lifespan of the short-lived rotifer *Asplanchna brightwelli* could be influenced by light or dark conditions. Very few studies of this type have been carried out. Erk and Samis (1970) and Pittendrigh and Minis (1972) found that exposure to continuous light shortened the lifespan of *Drosophila*. Similarly, von Saint Paul and Aschoff (1978) report that exposure to continuous light decreased the lifespan of the blowfly *Phormia terraenovae*. In contrast, Allemand *et al.* (1973) found that continuous exposure to light did not alter the lifespan of *Drosophila*. In the zooplankton, Hairston (1976) found that the copopod *Diaptomus nevadensis* will survive under continuous visible light only if it contains a high concentration of carotenoid pigment which serves as a photoprotective device.

The only study in which the effect of continuous darkness on lifespan was tested was that of Allemand *et al.* (1973). These investigators found that the lifespan of *Drosophila* was considerably extended by continuous dark conditions. Lifespan increased 20% for male and 43% for female *Drosophila* under conditions of total darkness.

The influences of light or dark conditions are of interest in biological gerontology because of the hypothesis discussed by von Saint Paul and Aschoff (1978) that lifespan is not determined by real time, but by revolutions of the circadian clock. Although the data presented on blowflies by von Saint Paul and Aschoff (1978) does not support this hy-

pothesis, it is notable that very little work has been done in this field and that the response of organisms other than insects to light or dark conditions has not been examined as a test of this hypothesis.

Rotifers were selected for this study since we are able to monitor their individual lifespans very closely (Verdone-Smith and Enesco, 1982a, b; Sawada and Enesco, 1984) and since they are known to respond to light. Several species of rotifers including *Asplanchna priodonta, Polyarthra remata* and *Filinia longiseta* exhibit positive phototactic reactions in the visible light and U.V. range (Menzl and Roth, 1972). This phototactic reaction is mediated by sensory pigments in the cervical eyespots of the rotifer (Cornillac *et al.*, 1983; Clement *et al.*, 1983). Evidence that rotifers respond to alterations of the light/dark cycle is provided by the studies of Pourriot and Clement (1975) who studied the mode of reproduction of the rotifer *Notommata copeus* in relation to photoperiod. *N. copeus* is parthenogenetic (amictic) in short-day conditions, but mictic females appear when the day length exceeds 14 hours.

In the present study the effect of LL (continuous light), the effect of DD (continuous dark) and the effect of LD 12:12 (control) were all examined to determine the extent to which light or dark conditions would influence the lifespan of the rotifer *A. brightwelli.* A circadian cycle of LD 6:18 was also imposed upon the rotifers to determine its influence on lifespan.

MATERIALS AND METHODS

The organism used in this study was the short-lived rotifer species, *Asplanchna brightwelli,* Clone 4B61. The rotifers were originally obtained from Dr. John J. Gilbert, Dartmouth College, Hanover, N.H. *Paramecium caudatum,* the food source of the rotifers, were cultured in a Cerophyll infusion medium with *Paramecium caudatum* and *E. coli* as indicated in a previous paper (Enesco and Verdone-Smith, 1980).

Groups of rotifers were exposed to four different light or dark conditions; continuous darkness (DD), continuous light (LL), LD 6:18, or LD 12:12 (control) cycle. The light source consisted of four 60W incandescent light bulbs (Canadian General Electric) and four cool white fluorescent lamps (No. F48T12/CW/SHO: Westinghouse) all placed approximately 45 cm away from the culture flasks. The light source was within a temperature controlled incubator set at 19.0 ± 0.5 °C.

Those rotifers which were maintained in total darkness were kept in three 250 ml Erlenmeyer flasks covered with black plastic coating and with aluminum foil. These flasks had an aluminum cap on top of the sponge stopper, which prevented light from entering at the top but allowed for exchange of gases.

Transfer and feeding of the rotifers grown in total darkness required great care since the manipulations were carried out in the dark. The flasks were first swirled and then 30.0 ml of medium containing rotifers was transferred using a macropipetter (Oxford 10 ml Macro-Set) into another plastic coated 250 ml Erlenmeyer flask containing 150 ml of fresh medium. The control groups were grown and transferred in the same manner as those grown in total darkness, except that the Erlenmeyer flasks were not covered with aluminum foil or dark plastic and the transfer of the control groups was carried out in the presence of light.

The LD 12:12 and LD 6:18 light conditions were maintained by placing the rotifer cultures in incubators with timed light controls. The temperature, as for all experiments, was maintained at 19.0 ± 0.5 °C. Those rotifers in LD 12:12 or LD 6:18 conditions were maintained in uncoated Erlenmeyer flasks.

After a calculated minimum of 20 generations in the respective light conditions, rotifers from each group were individually segregated into the wells of 24-welled tissue culture containers (Flow Laboratories) containing 2.5 ml of fresh medium in each well. Lifespan and fecundity data for each rotifer were recorded every 3 hours. The sub-cultures of 24 rotifers each were maintained in their respective original conditions. Every 6 hours, 1 ml of old medium was replaced with new medium to maintain a constant supply of food. During the observation period, the DD cultures were exposed to light for the minimum time needed to collect data. These observation periods did not exceed 36 min. per day.

Ingestion of Paramecia

Ingestion of paramecia was detected by allowing the rotifers to ingest paramecia exposed to 0.75 ug/ml of neutral red for at least 12 hours. This concentration readily dyed the paramecia. After the stained paramecia were ingested, the dark red stain could be seen in the gut of the rotifers. Rotifers were examined after a 5 hour

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exposure to the dyed paramecia. The concentration of 0.75 ug/ml neutral red produces clearly visible gut coloration without any adverse effects on the lifespan or fecundity of *A. brightwelli* (Sawada, 1983; Sawada and Enesco, in press). Rotifers under the different light conditions were evaluated for their level of *Paramecium* ingestion at 3 different ages; 0.5, 2.5 and 4.5 days of age.

Statistical Analyses

One-way analysis of variance followed by the *post hoc* Tukey test was performed as indicated in Brunning and Kintz (1977) and Sokal and Rohlf (1981) to determine whether there were significant difference in lifespan or fecundity as a result of the light or dark conditions employed in this study. Data are presented with the standard error of the mean.

RESULTS

Table 1 shows the mean lifespan and maximum longevity of the rotifers reared in LL, DD or in a LD 12:12 cycle. A one-way analysis of variance showed that there were significant differences between the mean lifespans of the different groups ([F 2, 69] = 17.971, $p < 0.001$). Subsequent analysis using the *post hoc* Tukey test revealed that the mean lifespan of the DD group (6.56 ± 0.15 days) was significantly longer than the mean lifespan of either the LD 12:12 control group (5.56 ± 0.17 days) or of the LL group (5.25 ± 0.16 days). The mean lifespans of the LD 12:12 and the LL groups were not significantly different. Table 1 thus shows that the lifespan of the DD group was about one day longer than that of the other two groups.

Figure 1 shows the survivorship curves of *A. brightwelli* reared under the different light

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TABLE 1. MEAN LIFESPAN AND MAXIMUM LONGEVITY OF *A. BRIGHTWELLI* REARED UNDER LD 12:12, DD AND LL CONDITIONS. (n = 24)

Light Conditions	*Mean Lifespan ± S.E. (days)*	*Max. Longevity (days)*
LD 12:12 (Control)	5.56 ± 0.18	7.13
DD	6.56 ± 0.15*	7.75
LL	5.25 ± 0.16	7.00

*alpha = 0.01

TABLE 2. REPRODUCTIVE PROFILE OF *A. BRIGHTWELLI* REARED UNDER LD 12:12, DD AND LL CONDITIONS.

Light Condition	*Average Prereprod. Time ± S.E. (days)*	*Average Reprod. Time ± S.E. (days)*	*Average Postreprod. Time ± S.E. (days)*	*Ave. Offspring No. per Rotifer*
LD 12:12	2.15 ± 0.11	2.94 ± 0.23	0.21 ± 0.08	6
DD	3.31 ± 0.08*	2.88 ± 0.19	0.35 ± 0.13	6
LL	2.38 ± 0.05	2.65 ± 0.16	0.23 ± 0.06	7

*alpha = 0.01

or dark conditions. The difference in survival patterns became apparent on days 4 to 5, as shown by the shift of the survivorship curve of the DD rotifers to the right. Since the curves are so clearly separated, the difference in lifespan cannot be explained by prolonged survival of only a few individuals.

In Figure 2, the age specific death rate is shown for the three groups of rotifers reared under the three different light/dark conditions. Note that the DD group had a peak death period at 6½ days. This is a half-day later than the control group, which had the highest death rate at 6 days. The LL group had its peak death rate much earlier at 5 days.

Table 2 shows the data which form the reproductive profile of *A. brightwelli* under the different conditions. The length of the pre-reproductive, reproductive and post-reproductive periods was determined as well as the average number of offspring for all three groups. A one-way ANOVA showed that there were significant differences as a result of the light conditions in the length of the pre-reproductive period ([F 2, 69] =

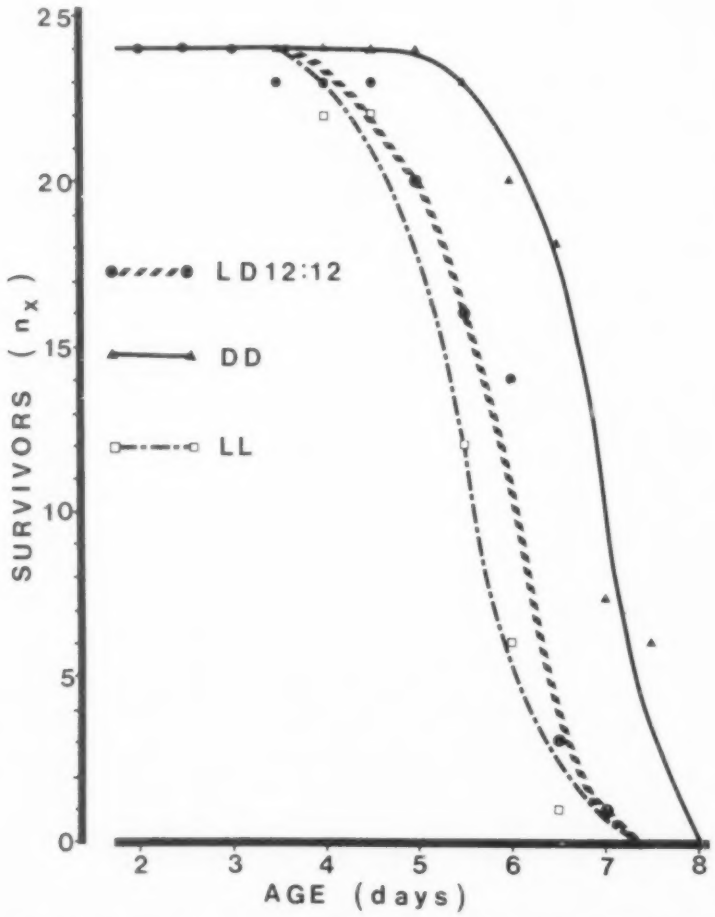

FIG. 1. Survivorship curves of *A. brightwelli* reared in LL, DD, or LD 12:12 conditions. (n = 24).

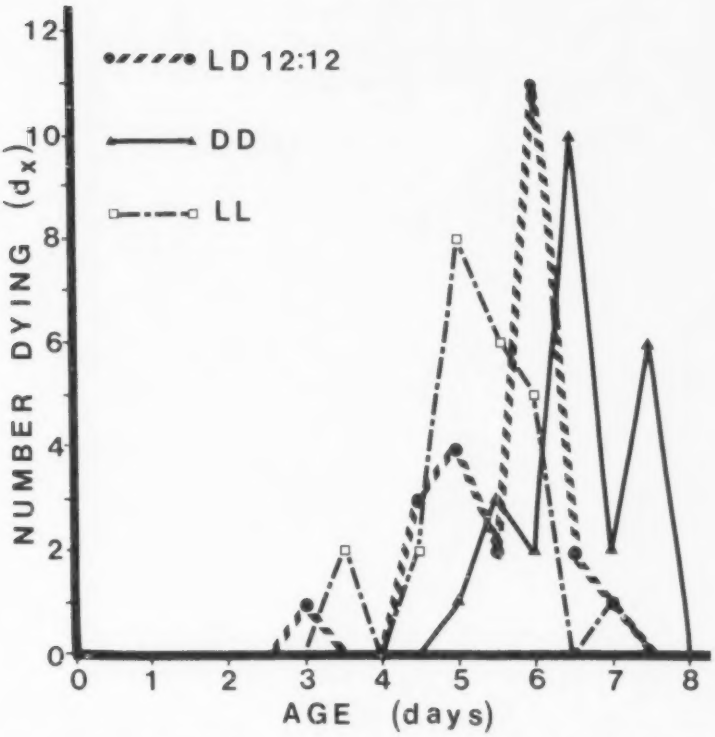

FIG. 2. The age specific death rates (dx) for *A. brightwelli* reared in LL, DD or LD 12:12 conditions. (n = 24).

51.564, $p < 0.001$) but not for the reproductive period ([F 2, 69] = 0.601, $p > 0.50$), post-reproductive period ([F 2, 68] = 0.678, $p > 0.50$) or for the average offspring number per rotifer ([F 2, 69) = 2.209, $p > 0.10$). The results of the *post hoc* Tukey test subsequently showed that the mean length of the pre-reproductive period (3.31 ± 0.08 days) was significantly longer for the DD group than for any other group (alpha = 0.01).

Experiments were carried out to check whether paramecia were ingested to the same extent by rotifers reared under the different light conditions (Table 3). In all cases the rotifers ingested the paramecia to an equal extent. That is, an equal percentage of rotifers in all groups had ingested the dyed paramecia after 5 hours of exposure.

In the next experiment, a subgroup of rotifers in DD conditions was transferred back to a LD 12:12 cycle to determine whether the prolongation in lifespan under DD conditions was fixed or reversible. This experiment was initiated several months after the previous experiment. This means that the rotifers had now been maintained in DD conditions for at least 50 generations.

The results on Table 4 show that the transfer from DD to LD 12:12 conditions caused a significant decrease in the lifespan of the rotifers. However, the lifespan of the transferred group was no different from that of the LD 12:12 control group.

It will be noted that the lifespan of both the LD 12:12 and of the DD groups were shorter in the second group of experiments (Table 4) than in the first group of experiments

TABLE 3. INGESTION OF NEUTRAL RED STAINED PARAMECIA BY *A. BRIGHTWELLI* AT DIFFERENT AGES AND UNDER THREE LIGHT CONDITIONS ARE SHOWN AFTER FIVE HOURS. (n = 48)

	% Rotifers With Dark Red Spot In Gut		
Age (days)	*LD 12:12 Control*	*DD*	*LL*
0.5	91.7	91.7	93.8
2.5	95.8	95.8	97.9
4.5	97.9	100.0	91.7

TABLE 4. THE EFFECT OF TRANSFERRING ROTIFERS FROM DD TO LD 12:12 CYCLE ON LIFESPAN. (n = 24)

Experiment	*Mean Lifespan ± S.E. (days)*	*Ave. No. Offspring*
LD 12:12 Control	4.81 ± 0.19	6
DD Control	5.46 ± 0.11*	7
DD to LD 12:12 Transfer	4.25 ± 0.22	4

*alpha = 0.01 from other 2 groups.

TABLE 5. THE EFFECT OF CIRCADIAN 6 HOUR LIGHT/18 HOUR DARK UPON LIFESPAN. (n = 24)

Experiment	*Mean Lifespan ± S.E. (days)*	*Ave. No. Offspring*
LD 12:12	5.47 ± 0.15	7
DD	6.41 ± 0.18*	8
LD 6:18	6.65 ± 0.19*	7

*alpha = 0.01 from LD 12:12 Control.

(Table 1). However, the standard error of mean is close to 0.2 days for both groups. Minor differences in the paramecia batchs and minor seasonal variation occur even under laboratory conditions. We occasionally observe that the control values deviate from one experiment to the next (Sawada, 1983).

In the next experiment we examined the effect of altered circadian cycle on rotifer lifespan. The lifespan of rotifers on a LD 6:18 cycle was compared with that of rotifers on DD and on LD 12:12 cycles. Table 5 shows that the life-span of both the rotifers on DD and the LD 6:18 cycles was significantly longer (alpha = 0.01) than that of control rotifers (on the LD 12:12 cycle). There were no significant differences in number of offspring between any of these groups. The survival curve for the rotifers on the LD 6:18 cycle was virtually identical to the survial curve for the rotifers in the DD conditions shown in Figure 1.

DISCUSSION

The results of the first series of experiments presented here show that the lifespan of the rotifer *A. brightwelli* is significantly extended following exposure to continuous dark (DD) as compared to control rotifers maintained in LD 12:12. On the other hand, exposure to continuous light (LL) did not significantly alter the lifespan of the rotifers. The findings that continuous darkness significantly extends lifespan is in agreement with the findings of Allemand *et al.* (1973) who found that DD conditions significantly extended the lifespan of *Drosophila melanogaster,* while LL conditions did not affect *Drosophila* lifespan. Our results differ from the findings of von Saint Paul and Aschoff (1978) who report that LL conditions reduced the survival of blowflies by 52 to 75%, depending on light intensity.

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In attempting to explain why *Drosophila* might live longer in DD conditions, Allemand *et al.* (1973) suggest the hypothesis that the increased longevity may be due to a reduction of activity in the flies, due to their inability to fly in the darkness. They rejected this hypothesis due to a lack of evidence relating metabolic rate to lifespan in insects. However, more recent studies clearly link activity levels with lifespan in insects. For example, Ragland and Sohal (1973, 1975) have shown that the average maximum lifespan of the housefly can be doubled by restriction of flight activity. Buchan and Sohol (1981) have shown that the lifespan of flies is inversely related to their level of physical activity, as measured by walking and flying activity.

We considered the question of whether rotifers reared in DD conditions might have a lower metabolic activity than rotifers reared in LL. There is, of course, in these studies no variable such as absence of flight which reduced metabolic rate in the *Drosophila.* Rotifers move and feed continuously in their aquatic millieu. Since rotifers detect their prey by touch and have no recognition of form or movement (Clement *et al.,* 1983) it did not seem probable that dark conditions would interfere with their feeding patterns. Nevertheless, we conducted a control experiment to compare the level of paramecia ingestion in rotifers exposed to the different light or dark conditions. Since neutral red stained paramecia could be detected in the gut of rotifers raised under DD, LL and LD 12:12 conditions to an equal extent, we can conclude that DD rotifers were not impaired in their ability to feed. Indirectly this showed that their level of movement, required to feed, was not altered by light conditions.

In other studies (Verdone-Smith and Enesco, 1982; Sawada and Enesco, in press)

we had established that dietary restriction by means of reduced feeding extends the lifespan of *A. brightwelli*. Since the DD rotifers in the present study were feeding to the same extent as the LL or LD 12:12 rotifers, we are able to eliminate dietary restriction as a cause of life extention in the DD group.

Our results show that there was no difference in the number of offspring produced by rotifers in DD, LL or LD 12:12 conditions. The number of offspring that this species of rotifer produces can be significantly reduced either by low temperature or by dietary restriction (Verdone-Smith and Enesco, 1982), both of which would slow metabolism. The fact that the number of offspring was not reduced by DD conditions thus argues against a metabolic change under DD conditions.

The length of the pre-reproductive period was significantly extended in the rotifers in DD conditions, while the length of the reproductive period and the length of the post-reproductive period remained constant. Of all known factors that cause a significant increase in lifespan, the lengthening is always in the pre-reproductive period. These factors which delay maturity include low temperature (Verdone-Smith and Enesco, 1982) and vitamin E administration (Sawada and Enesco, 1984). The conclusion of Stearns and Crandall (1981) that delayed maturity can reduce the cost of reproduction and increase adult survival rates is of interest in this context.

In a further attempt to understand the biological basis of the increased lifespan of the rotifers under DD conditions, we considered what might happen to lifespan if we shifted DD rotifers back to LD 12:12 conditions. If lifespan remained constant after the shift, the explanation could be in genetic drift. The DD group was started from a group of 20 rotifers maintaned as a separate subpopulation for a minimum of 20 generations; long enough for some degree of selection or drift to alter the genetic character of the population. If, on the other hand, the lifespan of the DD rotifers shortened when shifted to LD 12:12 conditions, the lengthening of lifespan would have to be interpreted as a dark adaptation. In fact, the results show that transfer of rotifers from DD to LD 12:12 conditions resulted in a significant decrease in lifespan. In fact, there was no significant difference in the lifespan of the newly transferred rotifers and control rotifers maintained continuously in our laboratory under LD 12:12 conditions. Thus we have established that the increase in lifespan observed under DD conditions is not the result of genetic selection, but is a temporary and reversible adaptation to dark conditions.

The question remains as to why dark conditions would lead to a significant increase in the lifespan of *A. brightwelli*. The answer may be that light, either in LL or in LD 12:12 conditions is a stress for the rotifers at the level used in laboratory conditions. *A. brightwelli* is quite transparent and does not appear to have pigments which would protect it against light. Hairston (1976) has shown that continuous light is a stress which significantly reduces the lifespan of copepod populations which lack the protection of carotenoid pigments. Similarly, Mathews and Sistrom (1959) have shown that mutant bacteria lacking carotenoid are killed by sunlight, whereas normal strains are unaffected. It is known that photo-oxidation may cause damage to sensitive molecules: damage may be medicated not only by U.V. light by visible light as well. Rotifers must not be excessively sensitive to light because their lifespan was no different in LL and LD 12:12 conditions. Nevertheless, the light could act as a stress.

An alternative interpretation as to why DD conditions significantly increased lifespan of the rotifers is that the shift to DD conditions was perceived as a disengagement from a circadian cycle. This explanation is supported by the last series of experiments. Here

rotifers shifted to an LD 6:18 cycle also showed a significant increase in lifespan. The rotifers in DD conditions showed an 18% increase in lifespan, while the rotifers in LD 6:18 conditions showed a 22% increase in lifespan over LD 12:12 controls. It should be noted that the rotifers in DD conditions had to be exposed to light for a minimum of 36 minutes per day during the actual lifespan determinations, although they were reared for a minimum of 20 generations under total darkness before use. Exposure to a shortened light period during lifespan studies was the common factor for both groups which had significant increases in lifespan. The marked increased in rotifer lifespan reported here may indeed be mediated by alterations in circadian rhythm.

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PLASMA TESTOSTERONE LEVELS ARE HIGHER IN OLD MATING RATS THAN IN OLD NON-MATING RATS

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(*Received* 26 *March* 1984)

Abstract—About one-half of 96 aged rats mated at 20 months of age. Their plasma LH and prolactin concentrations were similar to those in non-mating old rats, but their plasma testosterone concentrations were significantly higher. Exogenous testosterone administration failed to change the sexual status of the non-mating group.

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INTRODUCTION

TESTOSTERONE LEVELS are significantly lower in aged male rats, both in peripheral (Frankel, 1981; Frankel and Mock, 1981) and in testis vein plasma (Frankel and Mock, 1982). In previous reports from this laboratory, 27-month old male rats did not mate, nor did their hormones (testosterone, LH, prolactin) respond to an estrous female (Frankel, 1981) as is customary during mating in younger animals (Kamel and Frankel, 1978). If an old animal did occasionally mate, his hormones did respond to mating, and his basal testosterone levels were slightly higher than those of non-mating old rats (Frankel and Mock, 1981). Thus, the issue of testosterone concentration remained alive as a possible factor in retention of sexual behavior in senescence, even though no correlation was established between testosterone levels and mating performance in mature male rats (Damassa *et al.*, 1977). We tested the possibility that a decline in sexual behavior is specifically linked to a unique deficiency in plasma testosterone concentration by studying rats at a chronological turning point in their sexual senescence. We set aside 96 male rats when they reached 18–20 months of age, studied their sexual behavior and measured three hormones, testosterone, luteinizing hormone, and prolactin. At this age, approximately half were still mating. Of the three hormones, only testosterone was significantly different between the two groups.

METHODS AND PROCEDURE

Animals

Male Sprague-Dawley rats were reared in our laboratory, as previously reported (Frankel and Mock, 1981). They were maintained on a reversed light:dark cycle (lights off, 1000–2200 h). Purina rat chow and water were available *ad libitum*. Starting at three months of age, they were given sexual experience once a month. At 20 months of age, they were placed in semicircular plexiglass mating arenas, and their sexual behavior scored under

dim red light, as previously reported (Frankel and Mock, 1981). Females were brought into sexual receptivity by chronic subcutaneous implantation of 5-mm Silastic capsules containing estradiol benzoate and subcutaneous injection of 1.0 mg progesterone in sesame oil 4–6 h before use. Males were considered to be maters if within a 20-min period they demonstrated mounting and intromission; otherwise, they were classified as non-maters. In all cases, rats classified as maters intromitted at least twice, although in some cases, ejaculation did not take place in the 20-min period. Four days later, rats were anesthetized with ether, and blood was drawn by cardiac puncture within one min of picking up the rat from the cage. Their sexual behavior was then screened twice more in monthly intervals.

At two years of age, 5 of the non-mating rats were cannulated in their right jugular vein with the tip in the right atrium and placed in a cage equipped with a cannular flow-through swivel as previously described (Frankel and Mock, 1981). They were infused with 50 μg testosterone, which was dissolved in physiological saline to which 1% propylene glycol was added, over a one-h period by use of a Harvard pump (model 940) while a receptive female was in the cage. Their sexual behavior was monitored with the assistance of a computerized keyboard (Frankel, 1981).

At 25 months of age, 24 of the non-mating animals were selected and formed into two groups. The experimental group was administered 2.5 mg testosterone propionate sc daily for 10 days, and then 1.25 mg testosterone propionate daily for 4 more days. The control group was injected with sesame oil during the same period. Volume injected in both groups was 0.1 ml. At the end of the injection period, sexual behavior was monitored for 60 min (Frankel and Mock, 1981).

Hormone assays

Plasma was separated by centrifugation of the blood samples and stored at −20°C until analysis. Hormones were measured by radioimmunoassay; LH by the method of Niswender *et al.* (1968), and prolactin by use of the NIAMDD rat assay kit. Both methods were characterized in a previous report from this laboratory (Kamel *et al.*, 1977). Testosterone was measured by a radioimmunoassay developed in this laboratory (Frankel *et al.*, 1975). Hormone concentrations were compared between mating and non-mating groups by the Student *t* test.

RESULTS

At the time of the first screening of sexual behavior at 20 months of age, 44 animals were classified as maters, 48 as non-maters, based on the criteria already stated. Plasma LH and prolactin levels were similar in both groups (Table 1). However, plasma testosterone was significantly higher in the sexually active group ($p < 0.002$). The sexual behavior of the animals was screened twice more in succeeding months, and those rats who were consistent maters were compared with those who were consistent non-maters, using the same basal hormone concentrations measured the previous month (Table 1). At the second screening, there were 20 rats with a consistent mating record, compared to 24 rats who were consistent non-maters. At the third screening, the group of consistent

TABLE 1. HORMONAL LEVELS IN SEXUALLY ACTIVE AND SEXUALLY INACTIVE 20-MONTH OLD MALE RATS.

Animal Type (number of animals)	*Hormone Concentration (ng/ml)*		
	Testosterone	*LH*	*Prolactin*
Mating (44)	1.64 ± 0.15*	21.31 ± 1.23	26.33 ± 4.56
Non-mating (48)	1.06 ± 0.10	24.49 ± 3.22	21.42 ± 2.20

Sexually experienced male rats were 20 months old when screened for sexual activity. Only those rats which intromitted at least twice during a 20-min period with an estrus female were classified as maters. Four days later, blood was collected by cardiac puncture from ether-anesthetized rats. Plasma testosterone, LH and prolactin were measured in duplicate by radioimmunoassay; only testosterone was significantly different between the two groups (*$p < 0.002$).

maters was reduced to 13, consistent non-maters to 18. Only testosterone levels remained significantly higher in the mating group, as the two groups became more restrictive (second screening, testosterone concentrations were 1.764 ± 0.216 ng/ml [±SEM] in the mating group, compared to 0.843 ± 0.108 ng/ml in the non-mating group, $p < 0.0006$; third screening, 1.707 ± 0.30 vs. 0.897 ± 0.10, $p < 0.0006$). The comparison of LH and prolactin showed no change as the groups being analyzed became more restrictive, and the probability that they were different from each other remained non-significant.

By the time the rats reached 24–25 months of age, the non-maters infused with testosterone ($n = 5$) and the non-maters injected with testosterone propionate ($n = 12$) or vehicle alone ($n = 11$) showed no recovery of sexual function.

DISCUSSION

The involvement of plasma testosterone concentration in the decline of sexual behavior in aged rats is of course not surprising, but the complexity of the physiological arrangement is emphasized by the data. Even though the aged maters showed a very significant probability that their plasma testosterone levels would be higher than plasma testosterone levels in non-mating aged rats, the relationship of plasma testosterone levels and sustenance of sexual function is purely correlational. It is not possible to set forth an arbitrary plasma testosterone concentration, e.g. 0.7 ng/ml, as projected by Damassa *et al.* (1977) as a minimum concentration for normal sexual behavior in the male rat, and predict which old rats in our study would maintain their sexual function and which old rats would not. It is as if testosterone sustains sexual function in many different ways, at many different levels, so that behavioral and physiological criteria are affected differently (Hart, 1983). As the plasma testosterone concentration (reflecting testicular secretion of androgens) wanes, the various ingredients comprising sexual behavior decline unevenly. Thus we have a correlation with an imperfect predictive value. In addition the measure of plasma testosterone concentration is imperfect. A single measurement or a series of measurements usually neglects circadian and ultradian pulsations (Mock and Frankel, 1978: Karpas *et al.*, 1983) and any mediation of this concentration through hypothalamic receptors (Thomas *et al.*, 1982) is also neglected. Thus the plasma concentration is not completely representative of the testosterone concentration which may be most effective in the initiation and support of sexual behavior. In addition, it would be simplistic to cast testosterone in a unique role in senescence of sexual function. It is well known that castrated young rats continue to mate for some time after surgery (Davidson, 1966; Kamel and Frankel, 1979). The significant decrease in plasma testosterone levels in male rats does not occur when sexual behavior precipitously falls at 18–20 months of age. Instead it occurs at 7 months of age, when there is no indication that sexual behavior has faltered (Mock *et al.*, 1975; Frankel and Mock, 1982). It is also evident that reinforcement with exogenous testosterone fails to reverse the senescent decline in sexual function of aging male rats, despite earlier reports by Stone (1938) and Minnick *et al.* (1946). Although this result surprised us, it is fully corroborated by Gray *et al.* (1981) in their study of middle-aged rats. Nevertheless, when large numbers of old rats are studied, as we did in this report, it is evident that as a group, those rats with higher plasma testosterone levels will maintain sexual behavior longer than those with lower plasma testosterone levels. If we couple this result with earlier reports that in mature rats, non-maters are deficient in plasma testosterone concentration (Damassa *et al.*, 1977; Kamel *et al.*, 1977), it appears clear that plasma

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testosterone concentration is at present a critical measurement in the assessment of sexual behavior in the male rat, whether young or old.

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UNIQUE INCREASE OF SUPEROXIDE DISMUTASE LEVEL IN BRAINS OF LONG LIVING MAMMALS

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(*Received* 1 *March* 1984)

Abstract—A possible involvement of superoxide dismutase (SOD) level in determination of mammal's lifespan was examined by comparing six different enzyme levels including SOD in brains of eleven mammalian species of different lifespans. When the specific activities of the enzymes were plotted against lifespans on a linear scale, no or weak negative correlations were observed for all enzymes studied except SOD, which revealed positive correlation. The uniqueness of the SOD level suggests that it is one of the enzymes which evolved together with lifespans. It would contribute to long lifespan through protection against oxygen toxicity.

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INTRODUCTION

WHAT KIND of molecular mechanisms would be responsible for determining lifespans of different mammals? The fact that different species have their own specific lifespans indicates that the lifespan is under genetic control (Samis, 1978). On the other hand, comparative studies from evolutionary point of view suggest that the basic mechanism for creating new species resides in a change of regulatory genes rather than that of structural ones (Wilson, 1976, Wilson *et al.*, 1977, Ohno, 1983). Then the question is what kind of regulatory change would result in different lifespans among different species. One candidate which might give a longer lifespan to a species than others is a more efficient protection system against intrinsic as well as extrinsic damages. Recently we have shown that superoxide dismutase (SOD), which is supposed to work for detoxication of oxygen radicals in cells (Fridovich *et al.*, 1978), has a weak but positive correlation with lifespans of different mammals (Tolmasoff *et al.*, 1980). However, since enzyme levels in general can differ between species because of many reasons during evolution (Wilson, 1976, Wilson *et al.*, 1979), and since the correlation of SOD to lifespan is very weak, the conclusion is not convincing as has been argued by Sullivan (1982). One of the crucial questions is whether or not the kind of weak correlation observed is unique to SOD or, rather, common to most enzymes when compared as a function of an animal's lifespan. The number of genes determining lifespan is speculated to be very small (Cutler, 1975) when compared

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to total number of genes (Lewin, 1974). Here we have compared six different enzyme levels in the brains of eleven mammalian species of different lifespans and found that only SOD shows positive correlation to lifespans.

MATERIALS AND METHODS

Brains from 11 mammalian species were collected fulfilling the following three conditions: (1) frozen within two hours after death of individuals (Tolmasoff *et al.*, 1980); (2) available from three or more individuals of a same species; and (3) donor's age could be estimated approximately so that samples were limited to those of sexually mature young to adult animals. The sources were the following: mice (WHT/Ht) were bred in our laboratory (Hewitt and Sakamoto, 1971); golden hamsters and cats were purchased from Nisseizai Co. Ltd. (Tokyo); brain tissues of rats, guinea pigs, rabbits, dogs, and sheeps were from Pel-Freeze Co. Ltd. (Rogers, AR); brains of pigs and bovines were from Nippon Zoki Co. Ltd. (Tokyo); and horse brains were from Dr. S. Kawai at Yamanashi Livestock Laboratory (Isawa, Yamanashi). All brains were frozen on dry ice and kept at −70 °C until use.

One half to one gram of cerebrum was homogenized in 1.5 to 3 ml of water at 0 °C and centrifuged at 14,000g for 15 min. The supernatant was used for enzyme assay. We did not pay attention to separate gray and white matter because of the following reasons: (1) When gray-matter-rich and white-matter-rich fractions were separated from bovine or pig brains and measured six enzyme levels, the differences were less than 30%, which was smaller than the variation between individuals; and (2) it was very hard to separate gray and white matters of small brains like mouse's and rat's. Attention was paid instead not to use exclusively one of the tissue regions. With small animals, a whole or half of the cerebrum was used. The enzymes examined were selected on the bases of: (1) presence in brain; (2) relative stability; (3) easiness in assay; and (4) variation in enzyme category. The enzymes were lactate dehydrogenase (LDH, EC 1.1.1.27), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), superoxide dismutase (SOD, EC 1.15.1.1), glutamic oxalacetic transaminase (GOT, EC 2.6.1.1), creatin phosphokinase (CPK, EC 2.7.3.2), and choline esterase (ChE, EC 3.1.1.7). Enzyme activity unit (U) was determined from a change of light absorbance per min at 25 °C under the conditions described in each enzyme assay kit of Boehringer Mannheim. Absorbance was read with Hitachi Spectrophotometer Model 204 (Tokyo) using 1cm thick cubette at intervals of every 20 sec to 2 min depending on rate of reactions. The catalogue number and absorption change measured were as following: LDH; #124915, $-\Delta A_{340}$, G6PDH; #124672, ΔA_{340}, GOT; #124397, $-\Delta A_{340}$, CPK; #124184, ΔA_{340}, and ChE; #124133, ΔA_{405}, where Δ and A mean a change per min and absorbance, respectively. The SOD unit was determined as 50% inhibition of pyrogallol (Wako Chem. Co. Ltd., Osaka) autoxidation as reported before (Marklund and Marklund, 1974, Tolmasoff *et al.*, 1980). Protein was quantitated by Bio-Rad Protein assay kit (Bio-Rad, Richmond, CA). Specific activity was determined as an average of 3 to 4 individuals. Maximum lifespans were taken from Biology Data Book (Altman and Dittmer, 1972).

RESULTS

Table 1 lists specific activities of six enzyme levels of 11 mammalian species studied together with maximum lifespans (MLS) recorded. Means, standard deviations, and the number of individuals tested were shown. They were plotted on linear scale in Figure 1. The maximum lifespan is the most reliable measure of species' lifespan (Comfort, 1979). Some points showed wide variations, which might result from differences in physiological conditions among individuals (Benjamin and McKelvie, 1978). However, when one takes overall trends, only the SOD level shows positive correlation with maximum lifespans. The correlation coefficient after linear regression analysis was 0.644. The other enzyme levels do not reveal any evident positive relation. These may be classified into two groups; one which has negative correlation with lifespan, GOT (−0.673) and ChE (−0.680), and the other which shows no obvious tendency, LDH (0.320), G6PDH (−0.151) and CPK (0.266), with the correlation coefficients given in parentheses. Since metabolic rate has a negative correlation with body weight or lifespan of mammals (Brauer, 1963, Tolmasoff *et al.*, 1980), the former two enzymes might be refractory of metabolic rate.

It is noted that the SOD level in the horse is somewhat low compared to the other

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TABLE 1. MAXIMUM LIFESPANS (MLS) AND SPECIFIC ACTIVITIES OF SIX ENZYMES IN BRAINS OF 11 MAMMALS.

Species	*MLS (yr)*	*LDH*	*G6PDH*	*SOD*	*GOT*	*CPK*	*ChE*
mouse *(Mus musculus)*	3.5	5.00 ± 0.49 (4)	0.124 ± 0.011 (4)	8.94 ± 0.78 (4)	4.59 ± 0.30 (4)	20.5 ± 0.9 (4)	0.563 ± 0.064 (4)
hamster *(Mesocricetus auratus)*	4	11.45 ± 2.62 (4)	0.074 ± 0.002 (4)	11.01 ± 0.45 (4)	6.44 ± 0.50 (4)	16.3 ± 2.3 (4)	0.502 ± 0.131 (4)
rat *(Rattus rattus)*	4.7	10.43 ± 3.26 (4)	0.143 ± 0.014 (3)	11.49 ± 1.32 (4)	6.57 ± 0.79 (4)	28.2 ± 0.7 (4)	0.580 ± 0.151 (4)
guinea pig *(Cavia porcellus)*	7.5	5.62 ± 0.91 (3)	0.272 ± 0.024 (3)	12.12 ± 0.72 (4)	3.76 ± 0.20 (3)	30.1 ± 1.5 (3)	0.381 ± 0.078 (3)
rabbit *(Oryctolagus cuniculus)*	13	6.10 ± 0.51 (4)	0.122 ± 0.020 (4)	13.98 ± 1.83 (4)	3.22 ± 0.38 (4)	27.3 ± 2.4 (4)	0.683 ± 0.133 (3)
dog *(Canis familiaris)*	20	11.29 ± 1.18 (3)	0.069 ± 0.006 (3)	13.20 ± 2.19 (3)	4.16 ± 1.42 (3)	24.4 ± 4.2 (3)	0.278 ± 0.078 (3)
Sheep *(Ovis aries)*	20	11.56 ± 0.80 (3)	0.050 ± 0.022 (4)	17.91 ± 0.96 (4)	1.38 ± 0.17 (4)	25.7 ± 3.7 (4)	0.250 ± 0.062 (4)
swine *(Sus scrofa)*	27	3.23 ± 0.54 (3)	0.135 ± 0.005 (4)	20.28 ± 1.83 (3)	3.07 ± 0.75 (4)	18.9 ± 3.2 (4)	0.165 ± 0.042 (4)
cat *(Felis catus)*	28	7.54 ± 0.75 (3)	0.031 ± 0.007 (3)	16.77 ± 2.19 (3)	3.88 ± 0.90 (3)	35.9 ± 1.3 (3)	0.456 ± 0.006 (3)
bovine *(Bos taurus)*	30	12.04 ± 2.35 (4)	0.039 ± 0.008 (4)	21.63 ± 1.74 (3)	2.36 ± 0.26 (3)	26.6 ± 5.5 (4)	0.259 ± 0.081 (4)
horse *(Equus caballus)*	46	13.32 ± 1.61 (3)	0.176 ± 0.035 (3)	14.10 ± 1.71 (3)	2.46 ± 0.41 (3)	26.6 ± 3.5 (3)	0.237 ± 0.039 (3)

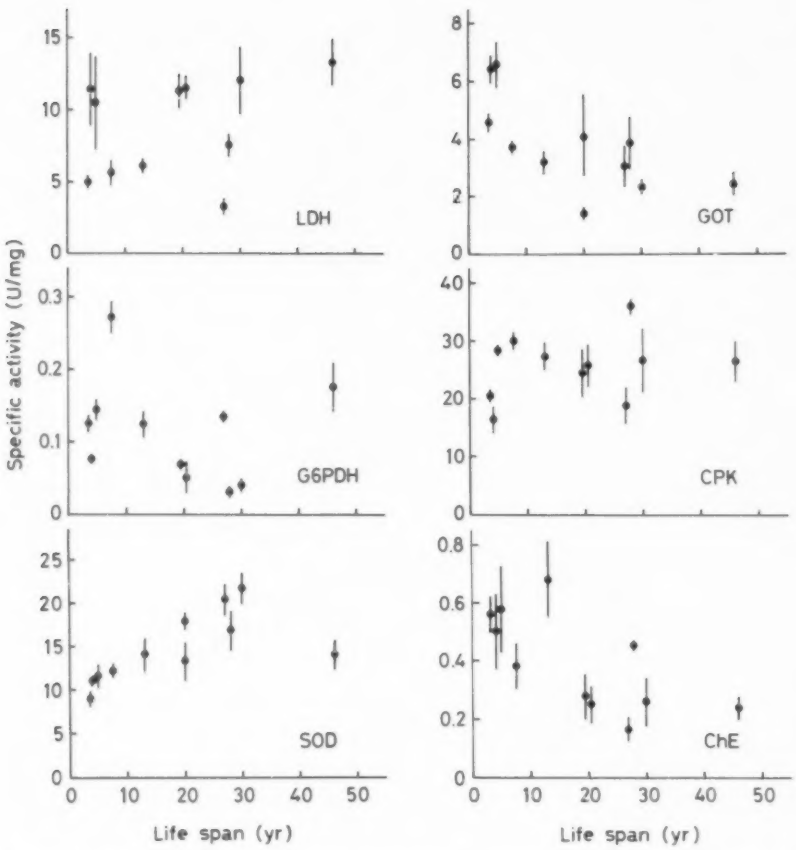

FIG. 1. Specific activities of six different enzymes in cerebrums of 11 mammalian species in relation to their maximum lifespans. Mean and standard deviation from 3 to 4 individuals were plotted.

values. However, the difference between mouse and horse is almost the same as that observed with some primate species of similar lifespans (rhesus monkey, olive baboon, and orangutan), which have been studied previously (Tolmasoff *et al.*, 1980). The other values are comparable to those in primate species of similar lifespan, that means the degree of deviation of SOD level from linear regression is not different between primates and non-primate mammals (*See* Discussion on this point).

DISCUSSION

A short note on the nature of comparative studies might be appropriate before discussing the result. Comparisons in general give circumstantial evidence and can hardly elicit a simple solid conclusion because one phenomenon is usually affected by plural factors. Physiological conditions of each individual can also have some influence on many aspects including enzyme levels. However, there remains a chance that properly controled ex-

amination reveals the factors responsible for a biological phenomenon. Especially when there are few clues to probe as in the case of lifespan study, comparative studies can be a powerful approach. A correlation between DNA repair capability and lifespan reported by Hart and Setlow (1974) is a good example, even though further investigations have revealed the relationship is not so simple (Kato *et al.*, 1980, Francis *et al.*, 1981).

SOD is one of the candidates which have been shown a possible involvement in determination of mammalian lifespan (Tolmasoff *et al.*, 1980). The present study has been conducted to see the uniqueness of positive correlation between SOD level and lifespan in comparison to the other enzymes. The six different enzymes, including five which were chosen at random, were measured with the same individuals of different species. To our knowledge, there have been no such study which would give a good comparison. The uniqueness of SOD observed suggests that the regulation of SOD level (keeping it at a high level) is involved in the maintenance of long lifespan. The mechanism would be the protection of cells from damage of superoxide radicals (Fridovich *et al.*, 1978).

The variation in relationship between SOD level and lifespan observed in mammalian species as well as in primates (Tolmasoff *et al.*, 1980) would imply that SOD levels and lifespan are not in a one-to-one relationship. Instead, SOD is one of the factors needed for long lifespan, which will be acquired by an evolution of many factors in concert (Cutler, 1975).

We thought in the previous studies (Tolmasoff *et al.*, 1980) that the ratio of SOD level to specific metabolic rate (SMR) should be compared rather than SOD level itself, for the protection efficiency would be inversely proportional to production rate of toxic oxygen, which would be parallel to metabolic rate. However, as was mentioned by Sullivan recently (1983), the variations of SMR among different animals were so large that the relatively small change of SOD level was suppressed when plotted in terms of SOD/SMR against lifespan. It made the relationship between SOD levels and lifespan obscure. The latest objection of Cutler (1983) against Sullivan's view was not quite convincing because SOD levels in the heart are a moot point. Our present results with SOD does not support Cutler's view if plotted in his way (data not presented).

Further studies are needed to confirm an involvement of SOD in lifespan determination.

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EUPLOIDIZATION OF HUMAN HEPATOCYTES FROM DONORS OF DIFFERENT AGES AND BOTH SEXES COMPARED WITH THOSE FROM CASES OF WERNER'S SYNDROME AND PROGERIA

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Abstract—Quantitative cytochemical studies of ploidy values for mono- and bi-nucleate hepatocytes from human livers of individuals from 15 weeks to 80 years of age, confirm earlier reports that the main ploidy shifts occur at 6–7 years, puberty and 21 years of age. In addition, the pattern of ploidy increase is similar to that described for other mammals.

Ploidy values obtained for hepatocytes from two patients with Werner's Syndrome are in excess of those from livers of normal individuals of similar age. In contrast, ploidy values from the liver of a single case of Hutchinson-Gilford Syndrome were similar to those from children of a similar age group.

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INTRODUCTION

A SHIFT from diploid to higher ploidy values is typical of mammalian livers (Gahan and Middleton, 1982a), such a shift appearing to be step-wise from mononucleate diploid ($1 \times 2c$) to binucleate diploid ($2 \times 2c$) to mononucleate tetraploid ($1 \times 4c$) to binucleate tetraploid and so on to higher ploidy values. A quantitative cytochemical study of ploidy values in hepatocytes from human livers of both sexes and covering an age range from 2 weeks to 90 years, has demonstrated a range of ploidy values which varies with age (Swartz, 1956). In particular, only diploid nuclei were observed in hepatocytes of individuals up to 6 years of age, DNA synthesis occurring in some nuclei between 6 and 10 years. By 11–14 years, a definite tetraploid class was established and until 20 years the livers contained diploid and tetroploid cells. At 20 years an octoploid class appeared, all three classes of nuclei existing until death. Unfortunately, no studies were made on the number of nuclei per hepatocyte and so it is not clear, for example, if all of the diploid nuclei were present in mononucleate cells or as a mixture of mono- and bi-nucleate cells. In consequence, the full significance of some of the data concerning the degree of polyploidy is not clear, especially for livers from very young or very old individuals.

The present study attempts to re-assess ploidy values and nuclear numbers in human hepatocytes, and the data are compared with results obtained from livers of two patients suffering from Werner's Syndrome and one patient with Hutchinson-Gilford Syndrome.

MATERIALS AND METHODS

Human liver material was obtained at autopsy and was kindly provided by Dr B.D. Lake (Hospital for Sick Children, London, U.K.) and by Dr F.I. Filipe (Guy's Hospital Medical School, London).

The liver samples from the two patients suffering from Werner's disease (both females aged 52 years) were kindly given by Dr G. Martin, University of Washington, USA, and that of the Progeria subject (4 years old, male) by Dr J.P. Shutt, Brooks Memorial Hospital, New York, USA.

Sections were cut from liver fixed in 4% formaldehyde and embedded in paraplast at a thickness of 14 μm, in order to have a high proportion of relatively intact nuclei in each section (Carriere and Patterson, 1962). Sections were dewaxed and reacted for the Feulgen test for DNA which was measured using a Vicker's M86 integrating microdensitometer (Middleton and Gahan, 1979). One thousand nuclei were assayed for each specimen, the number of nuclei per cell also being recorded together with the ploidy value for each nucleus.

RESULTS

The population of diploid nuclei can already be resolved into two groups of mono- and bi-nucleate cells as early as at 15 hours after birth (Table 1). The low level of mononucleate tetraploid cells would seem to represent hepatocytes in late S or G_2 phase of

TABLE 1. DISTRIBUTION OF THE HEPATOCYTE POPULATIONS IN DIFFERENT PLOIDY GROUPS, AND WHICH DISTINGUISHES BETWEEN MONO- AND BI-NUCLEATE CELLS FROM HUMAN LIVERS AT DIFFERENT TIMES AFTER BIRTH.

	% PLOIDY					
AGE	*1 × 2c*	*2 × 2c*	*1 × 4c*	*2 × 4c*	*1 × 8c*	*2 × 8c*
5 hours	93.9	5.4	0.6	0.1	–	–
3 weeks	93.5	5.8	0.5	0.2	–	–
1 month	95.4	4.3	0.3	–	–	–
11 months	92.0	7.5	0.5	–	–	–
1 year	95.3	4.4	0.3	–	–	–
2 yr 1 mth	95.0	4.8	0.1	0.1	–	–
2 yr 5 mth	95.6	4.1	0.3	–	–	–
3 yr 3 mth	93.3	6.5	0.2	–	–	–
3 yr 8 mth	92.6	6.9	0.5	–	–	–
3 yr 9 mth	91.2	8.0	0.7	0.1	–	–
3 yr 10 mth	88.7	9.8	1.4	0.1	–	–
4 yr 4 mth	95.2	4.8	–	–	–	–
4 yr 10 mth	95.9	3.7	0.4	–	–	–
5 years	94.7	4.6	0.6	0.1	–	–
5 yr 10 mth	91.8	7.9	0.3	–	–	–
6 yr 9 mth	91.5	6.2	2.3	–	–	–
6 yr 9 mth	85.1	13.5	1.2	0.1	0.1	–
6 yr 11 mth	92.7	7.2	0.1	–	–	–
7 years	89.2	8.3	2.4	0.1	–	–
7 years	91.7	7.0	1.0	0.3	–	–
8 yr 8 mth	89.4	8.8	1.8	–	–	–
8 yr 9 mth	75.1	19.4	4.8	0.7	–	–
9 years	91.1	6.3	2.4	0.1	0.1	–
9 yr 5 mth	87.9	11.1	0.8	0.2	–	–
13 yr 1 mth	84.7	9.8	4.8	0.6	0.1	–
56 years	68.8	18.2	10.2	1.3	1.5	–
70 years	52.6	19.7	17.9	6.0	3.6	0.1
79 years	68.8	15.2	10.6	2.9	2.5	–
80 years	69.3	15.2	10.8	2.0	2.6	0.1

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TABLE 2. DISTRIBUTION OF THE HEPATOCYTE POPULATIONS IN DIFFERENT PLOIDY GROUPS AND DISTINGUISHING BETWEEN MONO- AND BI-NUCLEATE CELLS FROM LIVERS OF 3 CASES OF PREMATURE AGEING.

	% PLOIDY							
	1 × 2c	*2 × 2c*	*1 × 4c*	*2 × 4c*	*1 × 8c*	*2 × 8c*	*1 × 16c*	*2 × 16c*
WERNERS SYNDROME	1.9	1.4	54.1	29.5	9.1	2.5	1.4	0.1
WERNERS SYNDROME	2.0	1.5	48.6	28.3	14.8	2.9	1.7	0.1*
PROGERIA	95.5	4.3	0.2	–	–	–	–	–

*plus 0.1% 1 × 32c

a mitotic cell population. This state exists in all livers examined from either sex up to about 6 years of age. In addition, odd bi-nucleate tetraploid cells are present in some livers of this age group indicating that a capacity to shift to the octoploid state is already present in some individuals, although this is not pronounced before about 7 years of age when the 2 × 4c hepatocytes are accompanied by 1 × 8c cells between 7 years and puberty (Table 1). In the older livers, 2 × 8c cells also appear although a markedly high proportion of hepatocytes in livers from 56–80 years old donors remain diploid. By this period of life some 25–30% of all hepatocytes are tetraploid (either 2 × 2c or 1 × 4c) (Table 1).

Similar studies (Table 2) on the two samples of liver from cases of Werner's disease showed ploidy values closer to those found in normal liver tissue from 80 years old, and much changed from the 56 years old liver (Tables 1 and 2). This involved a sharp decline in 1 × 2c hepatocytes and a big increase in 1 × 4c, 2 × 4c and 2 × 2c hepatocytes, a feature not noted in livers from older individuals where the diploid cell population remained high.

In contrast, the liver from the case of Hutchinson-Gilford Syndrome showed a very juvenile ploidy distribution corresponding to that of the normal age group 15 hours–6 years (Tables 1 and 2), and with no sign of advanced polyploidization.

DISCUSSION

In general, there was little discrepancy between the ploidy values for hepatocyte nuclei from individuals of different ages reported by Swartz (1956) and in the present communication (Table 1). However, the assessment into mono- and bi-nucleate cells for each nuclear ploidy value has shown a series of steps from 1 × 2c to 2 × 2c, to 1 × 4c to 2 × 4c etc, state for each class as reported for other mammals (Gahan and Middleton, 1982 a,b). It is likely, therefore, that human hepatocytes will move to a higher ploidy state through a sequence of events similar to that reported for rats and mice in which bi-nucleate cells (2 × 2c) form from mono-nucleate (1 × 2c) cells, and give rise to mono-nucleate tetraploid cells (1 × 4c) by mitotic spindle fusion (James, 1977).

A comparison of liver cells from two cases of Werner's Syndrome (rapid ageing in adults) indicates a large shift to higher ploidy levels expected in very old individuals and differing from that seen in normal livers from a similar age group (Tables 1 and 2). In particular, the almost complete disappearance of the 1 × 2c and 2 × 2c cells is a factor which is strikingly different to other reported data for normal, aged livers (Tables 1 and 2; Swartz, 1956). Thus, whilst the trend is similar to that of aging, additional factors are in-

volved, resulting in the dramatic shift from lower to higher ploidy values, possibly resulting from the stimulus of an extra work load placed upon the liver (Gahan, 1977).

In sharp contrast, the Hutchinson-Gilford Syndrome (juvenile rapid aging) hepatocytes do not show signs of rapid aging, but are close to the data for normal liver from the 15 hours–6 years old age group (Tables 1 and 2; Swartz, 1956). These data are in agreement with the idea that Progeria does not necessarily represent a rapid aging syndrome (Gahan, 1973).

However, it could be argued that since the human liver does not normally show a dramatic shift to higher ploidy values before puberty, then this is a predictable result (Table 2). It might need the later activity of e.g. the anterior pituitary growth hormone to help trigger the shift to higher ploidy values which can be shown to be initiated by this hormone (Swartz, 1956; Helweg-Larsen, 1952; Leuchtenberger *et al.*, 1954).

Confirmation of these findings awaits the availability of more liver samples from Hutchinson-Gilford and Werner's cases.

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PLASMA AND PITUITARY CONCENTRATIONS OF LH, FSH AND PROLACTIN IN AGING MONGOLIAN GERBILS

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Abstract—Samples of plasma and pituitary homogenates collected from female Mongolian gerbils (3–4, 11–13 and 20–25 month-old) at various stages of the estrous cycle were analyzed by radioimmunoassay for luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin (Prl). Plasma and pituitary LH concentrations were similar in all three age groups. Plasma FSH concentrations tended to increase with age while plasma Prl concentrations remained unchanged. Pituitary concentrations of FSH and Prl were variable between the age groups depending upon the stage of the estrous cycle. The inability of older gerbils to produce young appears to result primarily from age-related changes occurring in the uterus rather than from alterations in the hypothalamic-hypophyseal complex.

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INTRODUCTION

THE MAJORITY of laboratory rodents have been shown to exhibit a decline in litter size with increasing age (King, 1916; Ingram *et al.*, 1958; Biggers *et al.*, 1962; Roman and Strong, 1962; Soderwall *et al.*, 1960; Parkening, 1982). The Mongolian gerbil (*Meriones unguiculatus*) appears to be an exception, since three laboratories (Marston and Chang, 1965; Adams and Norris, 1973; Arrington *et al.*, 1973) have found no significant variations in litter size with increasing parity. The number of animals beyond 400 days of age capable of littering, however, drops precipitously in comparison to younger females (Adams and Norris, 1973; Arrington *et al.*, 1973). Therefore, the mean reproductive lifespan of the female gerbil is less than half its mean lifespan (Arrington *et al.*, 1973).

The following study was conducted to determine if gonadotropin and prolactin (Prl) levels varied with age in an attempt to detect a possible relationship between these hormonal levels and age-related sterility.

MATERIALS AND METHODS

Female Mongolian gerbils (3–4, 11–13, and 20–25 month-old) raised at the University of Texas Medical Branch in Galveston were derived from stock originally purchased from Tumblebrook Farms, West Brookfield, MA. They were housed under controlled temperature (21–23 °C) and artificial lighting (14 h light/10 h dark-

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ness, lights on at 0500 hours CST) and provided with food (Formulab Chow No. 5008, Ralston Purina Co.) and water *ad libitum*. Their diet was supplemented once a week with fresh carrots and oatmeal. None of the 3–4 month-old gerbils had ever been mated, whereas all of the 11–13 and 20–25 month-old gerbils had produced at least one litter.

Vaginal smears, taken daily from individual gerbils were examined cytologically for 5–6 weeks immediately prior to the experiments. The 3–4 and 11–13 month-old gerbils exhibited 4–5 day estrous cycles. The 20–25 month-old gerbils were highly variable exhibiting 4–7 day estrous cycles. Some of the latter females had a leukocyctic (diestrous) smear for 2–3 continuous days, others had many cornified cells on the day of proestrus followed by a normal estrous smear and still others exhibited only a few cornified cells on a day typically considered to be estrus. Gerbils were killed on the first day they exhibited a prominent leukocyctic or a cornified cellular smear and this day represented the diestrous and estrous stages of the cycle.

At specific times of the estrous cycle, gerbils were bled unanesthetized by cardiac puncture with a heparinized 2 ml syringe (23-gauge needle). Enough blood for radioimmunoassay (RIA) was generally acquired within 2 min from the onset of bleeding. Once the blood was acquired the animal was killed by cervical dislocation and the pituitary gland removed, weighed and homogenized in 1.0 ml of 0.01 M phosphate-buffered saline (pH 7.6), containing 0.05 M EDTA and 0.1% sodium azide. Plasma and pituitary homogenates were stored at −85 °C until all of the samples were collected to permit their analysis in the same RIA. Sufficient plasma was acquired to allow for LH, FSH and Prl to be measured in each animal.

NIADDK rat materials were used for the RIA of plasma and pituitary LH, FSH and Prl. A standard double antibody RIA was used as described previously (Parkening 1980 a,b). The use of rat RIA materials for measuring each hormone was validated by comparing the parallelism of dose-response curves formed from varying quantities of gerbil plasma and pituitary homogenates with curves formed from the NIADDK rat reference preparations. Double 100 μl samples of plasma and double 100 μl (LH) or 200 μl (FSH and Prl) samples of a 1/20 dilution of the supernatant from pituitary homogenates were analyzed for each hormone. The concentrations of each hormone were expressed as ng equivalents of the reference preparations per ml plasma or μg pituitary wet weight. Reference materials were NIH-rLH-RP-1, NIH-rPRL-RP-1 and a gerbil plasma pool for FSH. All samples for each hormone determination were analyzed in the same RIA and the intraassay coefficients of variance were less than 10%. The sensitivities of the assays were 1 ng LH/tube, 1.8 ng equivalents FSH/tube and 0.2 ng Prl/tube.

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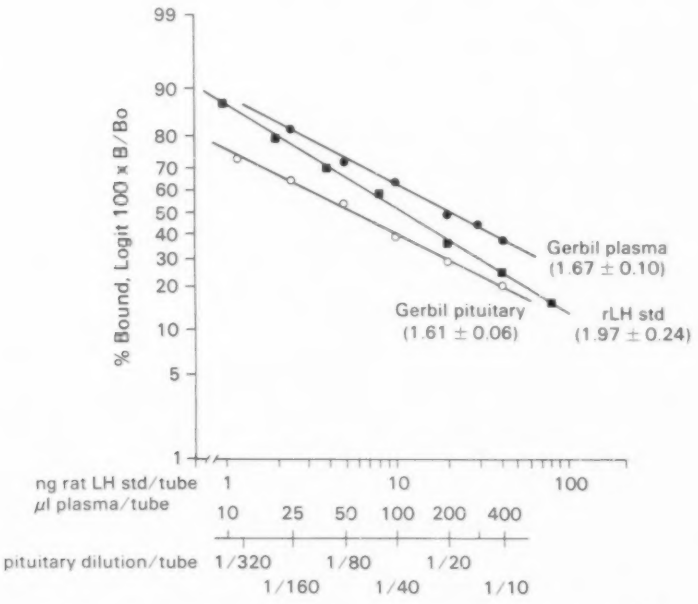

FIG. 1. Comparison of parallelism between the standard curve for NIADDK rat LH-RP-1, using NIADDK anti-rat LH serum and ^{125}I-rat LH and the dose-response curves of plasma and pituitary supernatants from Mongolian gerbils. The slopes of the lines are the means of four separate determinations.

The data was statistically analyzed using one-way analysis of variance, two-way analysis of variance and the Student *t* test. Differences were considered significant when $P < 0.05$.

RESULTS

The mean plasma dose-response curve for LH in the gerbil was found to parallel the mean rat LH standard curve (Figure 1). This was not true, however when comparing the homogenized pituitary gland of the gerbil with the rat NIADDK standard ($P < 0.005$). The dose-response curves for plasma and pituitary concentrations of FSH and Prl in the gerbil were statistically different ($P < 0.001$) from those of the rat NIADDK standard curves. The curves for FSH are shown in Figure 2, whereas those for Prl were presented earlier (Parkening *et al.*, 1980b).

Hormonal concentrations from blood plasma, pituitary blood plasma and pituitary homogenates acquired during the morning of each day of the estrous cycle were statistically compared by two-way analysis of variance. It was determined whether hormonal differences existed either because of age, the stage of the estrous cycle or the pattern of response through time (age-by-time interaction). Because the early evening samples were only taken on the day of proestrus, and since they were acquired only from the 3–4 and 20–25 month-old gerbils, these differences were determined by the Student *t* test.

Plasma and pituitary concentrations of LH were not statistically different as a function of age. Some differences ($p < 0.005$) were apparent, however, when examining samples taken during mornings of different days of the estrous cycle. There was a tendency for the

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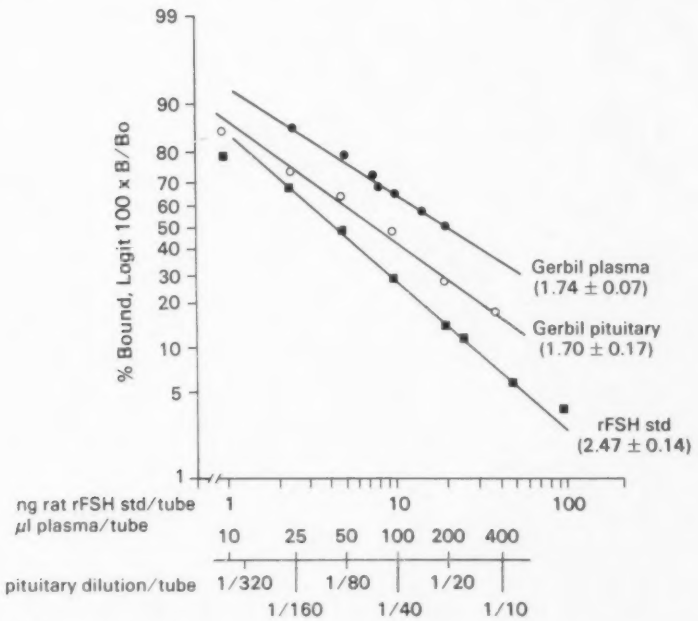

FIG. 2. Comparison of parallelism between the standard curve for NIADDK rat FSH-RP-1, using NIADDK anti-rat FSH serum and ^{125}I-rat-FSH and the dose-response curves of plasma and pituitary supernatants from Mongolian gerbils. The slopes of the lines are the means of four separate determinations.

TABLE 1. PLASMA AND PITUITARY CONCENTRATIONS OF LH, FSH AND PRL IN 3–4, 11–13 AND 20–25 MONTH-OLD GERBILS DURING VARIOUS TIMES OF THE ESTROUS CYCLE.

Stage of the estrous cycle	*No. of gerbils*	*Plasma (mean ± S.E.M.)*			*Pituitary (mean ± S.E.M.)*		
		LH (ng/ml)	*FSH (ng eq./ml)*	*Prl (ng/ml)*	*LH (μg/ml)*	*FSH (μg eq./ml)*	*Prl (μg/ml)*
Proestrus							
3–4 month							
1000–1200 h	9	15.2 ± 4.0	149.4 ± 29.6	25.2 ± 4.6	5.1 ± 0.4	4.5 ± 0.3	2.7 ± 0.2
1700–1900 h	5	48.0 ± 6.0	187.0 ± 21.6	28.9 ± 1.9	4.5 ± 0.5	4.5 ± 0.4	2.2 ± 0.3
11–13 month							
1000–1200 h	6	14.1 ± 1.8	189.8 ± 35.5	22.9 ± 5.6	6.2 ± 0.4	6.0 ± 0.4	2.7 ± 0.2
20–25 month							
1000–1200 h	8	15.2 ± 1.4	113.4 ± 26.1	22.2 ± 3.3	6.0 ± 0.3	6.1 ± 0.5	2.4 ± 0.3
1700–1900 h	8	43.1 ± 4.5	301.9 ± 57.0	18.0 ± 2.1	4.4 ± 0.4	3.2 ± 0.3	2.4 ± 0.2
Estrus*							
3–4 month	10	15.5 ± 2.9	190.0 ± 39.2	28.0 ± 2.6	5.1 ± 0.3	3.7 ± 0.5	2.0 ± 0.2
11–13 month	7	16.0 ± 3.5	206.6 ± 50.9	18.5 ± 3.7	5.0 ± 0.5	4.4 ± 0.3	2.2 ± 0.3
20–25 month	8	7.9 ± 1.0	557.5 ± 55.2	19.6 ± 2.6	5.8 ± 0.6	4.3 ± 0.4	2.1 ± 0.2
Metestrus*							
3–4 month	8	11.2 ± 1.4	378.3 ± 63.7	18.2 ± 1.5	4.8 ± 0.5	4.9 ± 0.4	2.3 ± 0.5
11–13 month	6	11.1 ± 0.7	404.8 ± 64.5	25.2 ± 4.0	4.9 ± 0.4	4.2 ± 0.4	3.2 ± 0.4
20–25 month	6	7.5 ± 2.2	393.3 ± 34.5	23.0 ± 5.2	5.3 ± 0.6	4.7 ± 0.4	2.3 ± 0.3
Diestrus*							
3–4 month	6	14.4 ± 2.3	164.6 ± 31.1	29.7 ± 4.2	5.1 ± 0.4	3.0 ± 0.6	2.7 ± 0.2
11–13 month	6	10.3 ± 2.5	209.0 ± 54.5	24.2 ± 3.2	4.9 ± 0.6	5.1 ± 0.5	2.8 ± 0.4
20–25 month	15	14.8 ± 1.3	237.1 ± 24.0	20.9 ± 3.5	4.9 ± 0.4	4.6 ± 0.4	1.8 ± 0.1

*All samples obtained between 1000–1200 h.

oldest gerbils to have lower concentrations of plasma LH during estrus and metestrus (Table 1). No differences existed in concentrations of pituitary LH during the various stages of the estrous cycle. There were no differences in the age-by-time interaction for plasma or pituitary LH. Plasma concentrations of LH were markedly higher ($p < 0.001$) during the early evening in both 3–4 and 20–25 month-old gerbils, when compared with morning samples, but there were no differences between samples as a result of age. The concentration of pituitary LH was lower ($p < 0.005$) during the evening than morning hours in 20–25 month-old gerbils.

Concentrations of plasma and pituitary FSH differed between age groups ($p < 0.005$) and stages of the estrous cycle ($p < 0.005$) (Table 1). There was also a difference in the age-by-time interaction ($p < 0.001$) for plasma FSH, but none for pituitary FSH. There were no statistical differences in concentrations of plasma or pituitary FSH in 3–4 month-old gerbils when comparing the morning and early evening hours. Plasma concentrations of FSH were higher ($p < 0.005$) and pituitary concentrations of FSH lower ($p < 0.005$) during the early evening for 20–25 month-old gerbils.

There were no differences in plasma or pituitary concentrations of Prl during morning hours of the estrous cycle, except for age differences ($p < 0.005$) in pituitary Prl (Table 1). No differences existed in Prl concentrations for the morning and early evening time intervals when examining gerbils of the same age. Differences did exist ($p < 0.005$), however, when comparing plasma Prl concentrations in 3–4 and 20–25 month-old gerbils during the early evening.

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DISCUSSION

The parallelism of plasma LH dose-response curves from the gerbil with the NIADDK rat LH standard confirms a previous finding by Turner (1972). The lack of parallelism of gerbil FSH, Prl and pituitary LH with NIADDK rat standards mean that the hormonal values obtained, while not quantitatively correct, still reflect any differences existing between the three groups. To quantitatively measure such hormones in the gerbil it will be necessary to develop a sensitive homologous radioimmunoassay for gerbil FSH and Prl as done recently for the radioimmunoassay of Prl in the Syrian hamster (Soares *et al.*, 1983).

It was apparent from this study that plasma concentrations of LH in 20–25 month-old female gerbils were very similar to those in younger gerbils. There was a tendency, however, for plasma FSH concentrations to increase with age. In premenopausal women, Reyes *et al.* (1977) showed that plasma FSH levels gradually rise several years prior to menopause. Serum LH levels in these same women did not show a similar rise. The aging gerbils in this study may be reflecting a similar type of gonadotropin secretory pattern. The high FSH levels may also be a reflection of the development of fewer ovarian follicles, which would otherwise produce sufficient quantities of inhibin to exert a negative feedback effect on the hypothalamic-pituitary complex. Plasma Prl levels remained similar during the estrous cycle except for evening values during proestrus. Plasma Prl concentrations in 5–6 month-old and 17–20 month-old Chinese hamsters at 1800 hours of proestrus were significantly different (Parkening *et al.*, 1981). However, when Prl values were determined for several time intervals during the evening of proestrus and the morning of estrus it became apparent that the episodic release of Prl was similar but not synchronized in the two age groups of hamsters. No statistical differences existed

when comparing plasma LH levels from 3–4 and 20–25 month-old gerbils at 1700–1900 hours of proestrus, although at 1000–1200 hours on the morning of estrus, LH levels in the oldest gerbils were approximately half those of 3–4 and 11–13 month-old animals. C57BL/6 mice show a progressive decline in LH during the proestrous surge with increasing age (Parkening *et al.*, 1982b). If samples had been taken at a greater number of time intervals during the LH surge, a similar pattern may have been evident in the gerbils. More complete studies are needed in the aged gerbil to explain the hormonal differences which were evident in this study.

The 20–25 month-old gerbils in this study were not capable of producing young, yet some were observed to mate and all continued to exhibit an estrous cycle. Eighteen of the oldest females in which vaginal smears were examined on a daily basis for 5–6 weeks exhibited blood in their smears for 1–5 days. While a laparotomy was not performed on any of these gerbils, it was presumed that they were pregnant and that the fetuses had died in utero and were being reabsorbed. All of these gerbils returned to a non-bloody vaginal smear and were eventually killed at various stages of the estrous cycle for this study. Histological studies of the ovaries were not done on any of the gerbils. Based on the above observations and the visible presence of developing follicles and corpora lutea on dissected ovaries of females killed during the study, the ovary of the oldest gerbils is still capable of ovulating. The incidence of cystic ovaries in this study was not as great as that reported for gerbils from another colony (Norris & Adams, 1972). Only 1 ovary from 38 (3%) 3–4 month-old gerbils were cystic compared with 5 ovaries in 25 (20%) 11–13 month-old and 6 ovaries in 45 (13%) 20–25 month-old gerbils. One gerbil in each of the two latter age groups had both ovaries cystic.

A comparison of data from the aging Mongolian gerbil with that of similar studies conducted on the C57BL/6 mouse (Banerji *et al.*, 1981; Collins *et al.*, 1980, 1981; Finch *et al.*, 1980; Parkening *et al.*, 1982a), Syrian hamster (Parkening *et al.*, 1982c,d) Chinese hamster (Parkening *et al.*, 1981, Parkening, 1982), Long-Evans rat (Huang *et al.*, 1976; Lu *et al.*, 1980), Wistar rat (Parkening *et al.*, 1982c), and Sprague-Dawley rat (Wise and Ratner, 1980) emphasizes the subtle differences detectable between such laboratory rodents when attempting to determine the primary cause(s) for reproductive senescence. Age-related changes influencing reproductive decline in the mouse, rat and Syrian hamster appear to be centered primarily in the hypothalamic-hypophyseal complex. Changes in the reproductive system of the aging female gerbil more closely resemble those of the aging Chinese hamster, suggesting the uterus may be largely responsible for the animals inability to beget young. Additional data is necessary, however, to make certain that oocytes in older gerbils are viable and that the corpora lutea in such animals are capable of supporting the early implanted embryos.

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EXPERIMENTAL TUMORS AND AGING: LOCAL FACTORS THAT MAY ACCOUNT FOR THE OBSERVED AGE ADVANTAGE IN THE B16 MURINE MELANOMA MODEL

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Abstract—In the B16 murine melanoma model tumor growth has been shown to be slower in animals of advanced age. One feature associated with this slower growth has been prominent fibrosis demonstrated in biopsies of the tumor in older animals. We have performed experiments to examine the fibrotic response in young and old mice. In non-tumor bearing animals the capacity to regain skin strength after surgical laceration and healing by primary intention was greater in old mice. Histologic preparations suggested a more prominent fibrosis at the wound site. The animals who were injected subcutaneously with B16 melanoma and treated with L 3, 4-dehydroproline (an inhibitor of collagen synthesis) local tumor growth was significantly enhanced only for the old animals. Although this inhibition of collagen synthesis produced a differential growth enhancement, there remained a significant difference in tumor volume between young and old animals. We conclude that fibrogenesis is an important host defense for containing local tumor growth and that this mechanism is preserved if not enhanced in mice of advanced age. Nevertheless other factors are needed to account completely for the observed age-advantage in the B16 melanoma model.

INTRODUCTION

THE PREVALENCE of neoplastic disease increases with age (Miller, 1980, Young, Asire and Pollack, 1976). Most likely this reflects a combination of circumstances including a failure of immune surveillance (Gatti and Good, 1970) and/or prolonged exposure to carcinogenic agents (Peto *et al.*, 1975). Of importance, the clinical course for elderly patients with certain tumors can be strikingly different from that of younger patients with histologically identical tumors. Although the general condition of the host and the coexistence of other diseases would portend a more ominous course for elderly patients, what is actually

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observed, at least for certain tumors (Ershler, Socinski and Greene, 1983; Sartoh, Shiramizu and Hida, 1982), is that slower tumor growth and fewer metastases occur in older people. To study this phenomenon experimentally, we have inoculated mice of varying ages with tumors and measured local tumor growth, metastases and survival. We found that older mice, given the same dose of a tumor (B16 melanoma) had slower tumor growth, a smaller final tumor volume and longer survival time (Ershler *et al.*, 1984). Furthermore, after intravenous (IV) injection the number of pulmonary metastatic colonies was fewer in the old mice. Histologic examination of the primary subcutaneous tumors revealed less vascularity, less necrosis and a more profound fibrotic component in tumors from the old mice. To extend these studies we have investigated the fibrous tissue and wound healing response in young and old mice, and correlated these with tumor growth. Our results indicate the presence of an exuberant fibrous tissue response in old mice which, when inhibited, results in enhanced tumor growth. It is apparent that local factors such as fibrous tissue response may account in part for the observed age advantage in this model.

MATERIALS AND METHODS

Mice and experimental tumor

Young C57 Bl/6 male mice (2–3 months old) were purchased from Charles River Laboratories, N. Wilmington, MA. Old C57 Bl/6 male mice were donated from the aging colonies of the National Institute on Aging maintained at Charles River Laboratories. For this strain of mice 2–3 months is considered sexually and immunologically mature (Altman and Katz, 1979) and 24 months is considered aged (Walford, 1976). The mice were housed at the animal care facility of the University of Vermont and were supplied food and water *ad libitum*. For each of the experiments a minimum of eight animals was allocated to each group.

The B16 murine melanoma was generously provided by Dr. Isaiah Fidler. In these studies the F10 subline of B16 was chosen. This subline is characterized by its increased colonization of the lung in experimental models of metastases (Fidler and Hart, 1982).

Tumor cells were stored frozen in RPMI 1640 (Flow Laboratories) with 10% V/V fetal calf serum and 7% dimethylsulfoxide (DMSO). The cells were thawed and grown in minimal essential medium (MEM) supplemented with 10% V/V fetal calf serum and antibiotics. For each experiment, cell viability was ascertained by trypan blue exclusion, and the cells were diluted to 5×10^5 viable cells/ml. For all experiments, 10^5 cells were injected subcutaneously (SC) into the right flank and animals were examined daily for survival and tumor growth. Palpable tumor was measured in two perpendicular axes with tissue calipers and the radius (r) estimated by dividing the mean diameter by 2. Tumor volume was calculated assuming spherical growth, by the formula $4/3\ \pi r^3$.

In each experiment sample tumors were resected, fixed in formalin, sectioned and stained with either hematoxylin-eosin or Masson's trichrome stain for microscopic examination.

The normal fibrotic response and wound healing. To determine if the degree of fibrosis in response to a stimulation other than tumor is different in young and old mice, we tested the wound disruption force in young and old mice. Animals were lightly anesthetized with ether and shaved. The skin was cleansed with absolute alcohol and an incision made with a fresh scalpel blade in the left paraspinal region on the dorsum of the animal 1.5 cm in length through the skin and the panniculus carnosus muscle. The wound was immediately closed with 3 skin clips applied at uniform distances. The animals were allowed to recover from their anesthesia and housed three per cage. Skin clips were removed on day 5 and the wounds analyzed at 14, 28 and 42 days.

Wound disruption strength was assessed by the method of Beckwith *et al.* (Beckwith *et al.*, 1963; Glaser *et al.*, 1965), with minor modifications. On the day of analysis, animals were sacrificed and rapidly skinned, harvesting the entire skin of the dorsum with dissection carried out in the loose areolar tissue beneath the panniculus muscle. The harvested skin was placed on a rubber block and kept moist with normal saline until subjected to mechanical testing. All skin was tested within one hour of harvesting. Specimens for testing were cut by a specially designed set of cutters (Beckwith *et al.*, 1963) which are orientated at right angles to the long axis of the wound. A normal (uninjured) skin sample was taken from the adjacent right paraspinal area for pair-wise comparison for each animal. The skin specimen was clamped in the materials tester at each end. One jaw was fixed and the other was moved with constant (but adjustable) speed. The degree of elongation and the load applied to

the strip was measured by bridges coupled to a differential transformer and a load transducer and continuously recorded on a digital readout as well as an XY plotter. In this way, a standard load deformation curve was obtained, and the maximum load (wound disruption strength) determined. For certain samples a portion of the wounded skin not used for mechanical analysis was placed in formaldehyde for subsequent histologic analysis.

Effects of L 3,4 Dehydroproline on the tumor growth

Recently it has been demonstrated that L-3, 4-dehydroproline (DHP), a proline analogue, retards the fibrogenesis induced by the anticancer drug bleomycin by inhibition of collagen synthesis (Kelley, Newman and Evans, 1980). Furthermore, mice treated with this compound have been shown to have more rapid tumor growth in experimental models (Hacker, Newman and Hong, 1983) presumably by the prevention of fibrous tumor encapsulation. In our experiments, groups of young and old mice, administered 10^5 B16 F10 cells subcutaneously (SC) in the right flank on day 1, were treated daily thereafter with SC injection of DHP (25 mg/kg) or 0.9% NaCl for 21 days. Measurement of tumor growth was undertaken as described above.

Statistical Analysis

Differences in tumor growth rates between experimental groups were analyzed for statistical significance by the Student's *t* test. In the wound stretching experiments, paired sample analysis was performed.

RESULTS

Assessment of the normal fibrotic response in a wound healing model

To offer a partial explanation for the apparent age advantage (i.e. reduced local tumor growth) in the B16 model, we proposed that local fibrous containment was more prominent in the older host. Collagen synthesis and fibrous tissue production, essential for both primary and secondary intention wound healing, was tested in normal, non-tumor bearing, young and old mice by measurement of wound disruption force. Whereas the force required to disrupt uninjured young skin was greater than that for old skin ($p < 0.05$), we found that in older animals skin strength approached baseline after injury at a faster rate than did young (Figure 1). At week six after laceration this difference is statistically significant ($p < 0.05$). Furthermore, histologic examination of randomly selected samples revealed more fibrous proliferation in samples from old mice (Figure 2).

DHP and tumor growth

If exuberant fibrous tissue proliferation did account for the restrained tumor growth apparent in aged animals, then inhibition of this response would be expected to abrogate the age difference in growth rate. We observed that tumor growth was significantly greater in DHP treated old animals when compared to saline controls, however the difference in growth rates between the two age groups was not completely abolished. We chose, for comparison purposes, to report the tumor volumes at day 21. By this date all animals, young and old, have measurable tumors, yet the largest tumors are not yet necrotic, and therefore have not reached the plateau phase of their growth curve. This observation "window" generally occurs between day 19 and 24 in these experiments. Representative biopsies are shown in Figure 3. Tumor volumes at day 21 are depicted in Figure 4. As can be seen, there is a slight increase in tumor volume (albeit not statistically significant) in the DHP treated young animals. The older animals, however, had a significant enhancement of tumor growth by the DHP treatment ($p < 0.05$). When this experiment was repeated similar observations were recorded.

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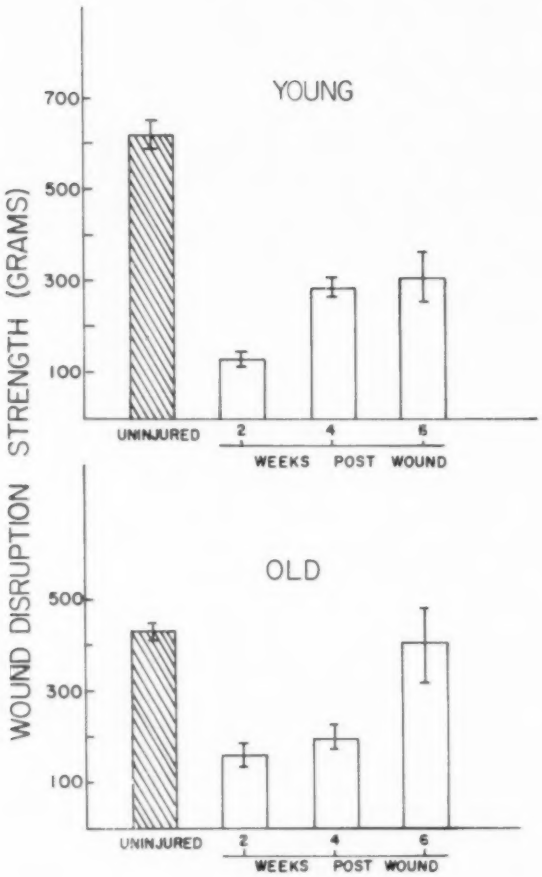

FIG. 1. Wound healing in young and old mice. Two, four and six weeks after surgical laceration animals were sacrificed and wound disruption strength was assessed. The force required to break normal uninjured skin was also determined for each animal and the mean ± SEM for all the young or old animals is represented by the hatched columns. The mean wound disruption forces (± SEM) at two, four and six weeks are represented by the open columns. Although old uninjured skin breaks with less force applied ($p < 0.05$), it is apparent that old healed wounds regain baseline disruption strength earlier ($p < 0.05$ at week 6).

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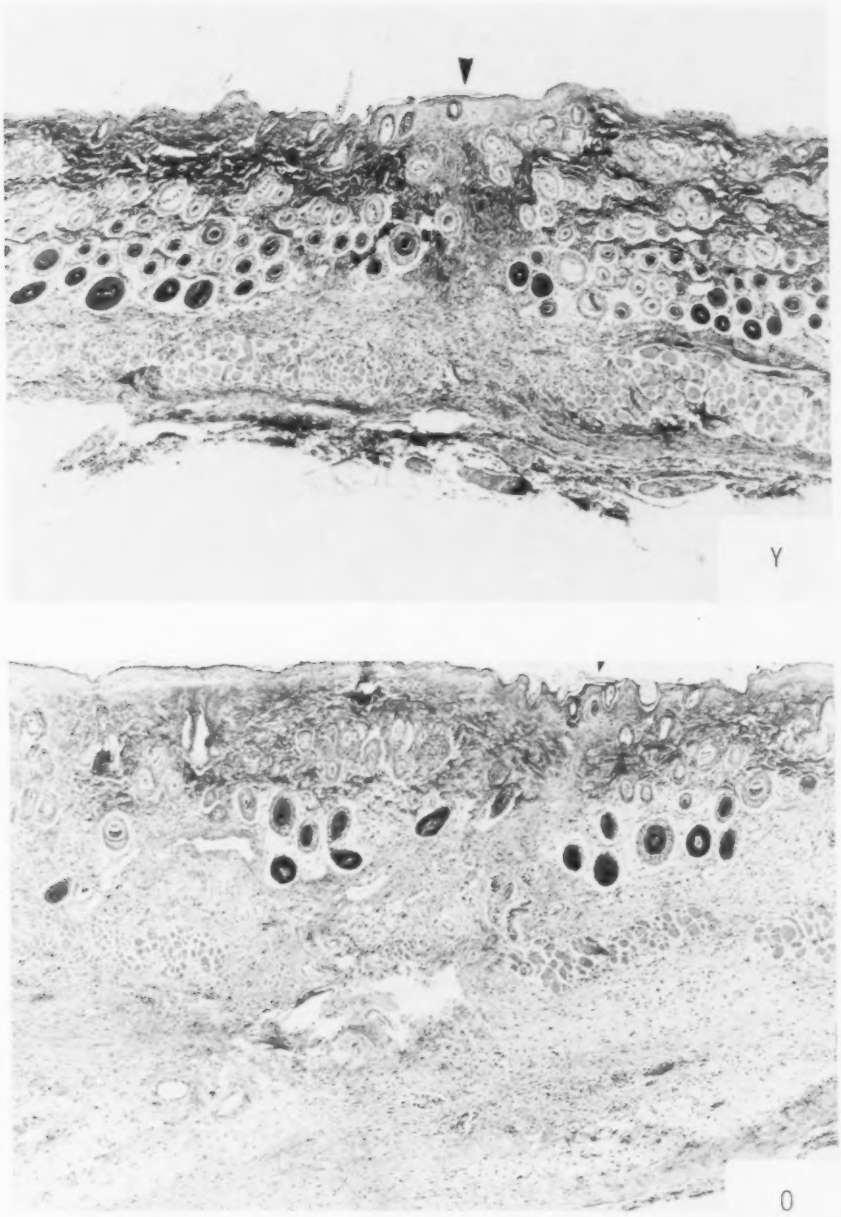

FIG. 2. Characteristic biopsies of healed wounds 6 weeks post laceration. The healed laceration is at the midpoint (arrow) and runs perpendicular to the long axis of the specimens. Both young (Y) and old (O) wounds appear intact, however there appears to be a greater fibrous component in the older wound. Masson's trichrome stain, ×40.

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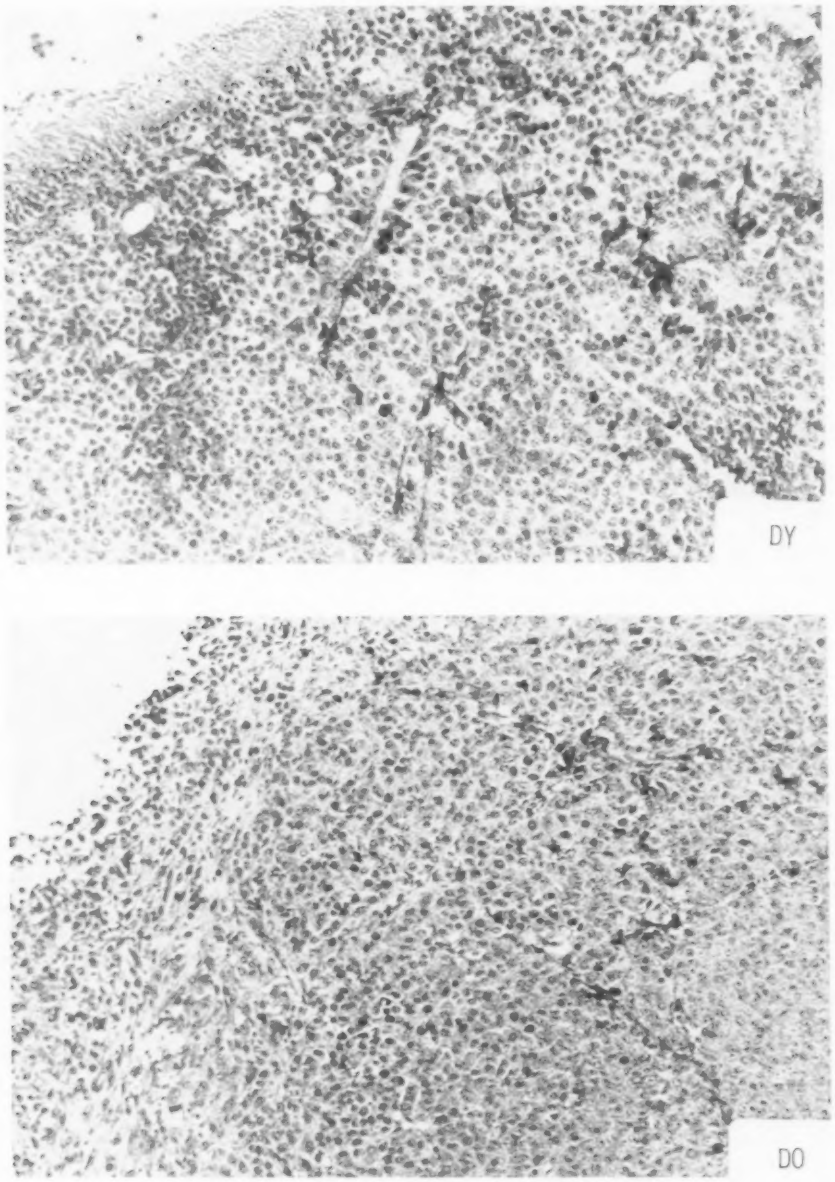

FIG. 3. Characteristic biopsies of tumors in DHP treated young (DY) and old (DO) mice and saline (SY and SO) controls. Fibrosis is prominent only in tumors from saline treated old animals. Also note the apparently greater vascular content in the tumors from the young animals. Masson's trichrome stain ×100.

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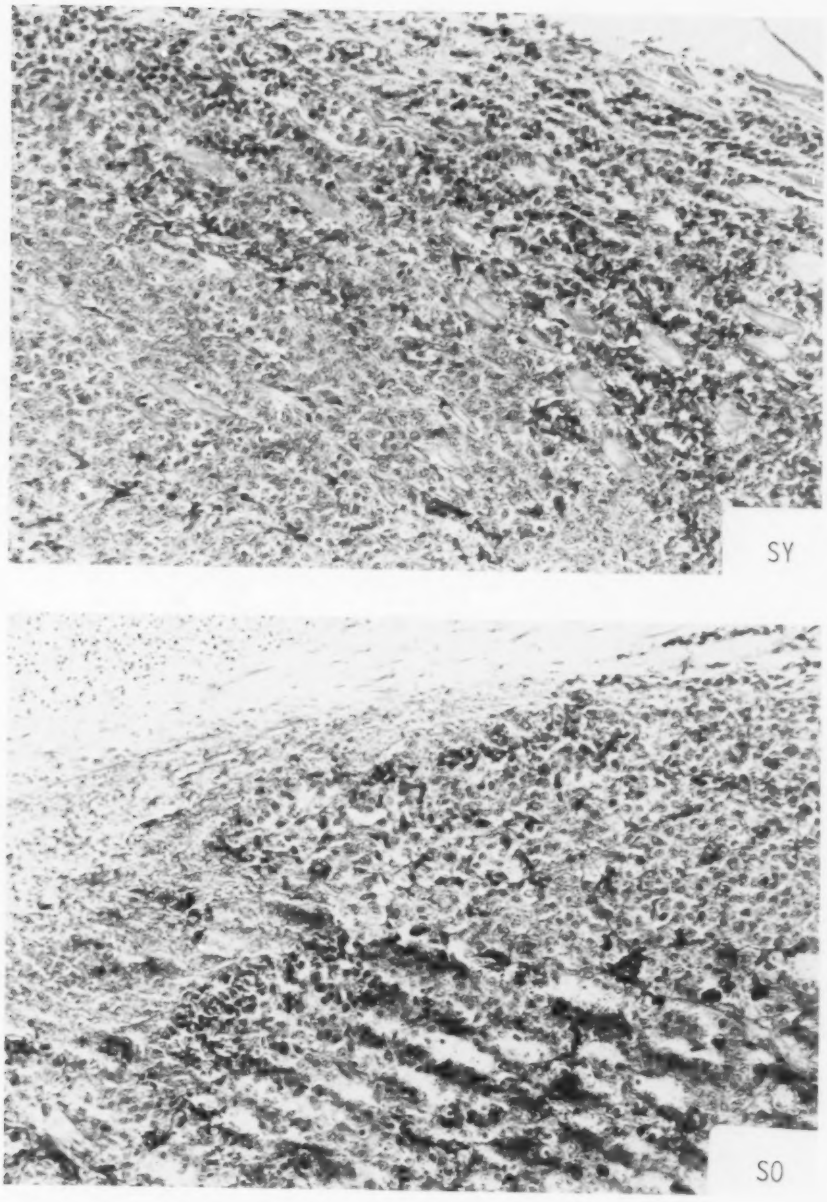

FIG. 3. (continued)

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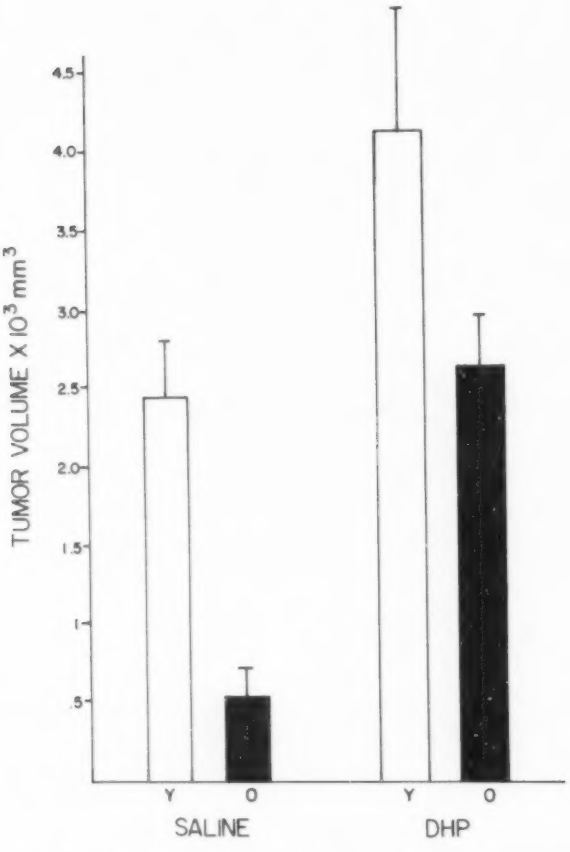

FIG. 4. B16 tumor volume in young (Y) and old (O) mice treated after tumor innoculation with either saline or DHP. Mice were treated with daily injection with either DHP or saline and tumor growth was recorded. Mean tumor volumes (± SEM) for each group of eight mice at day 21 is depicted. Tumor growth was greater in the young animals. DHP treatment however resulted in a significant ($p < 0.05$) enhancement of growth in the older hosts whereas there was no significant enhancement in the young animals. Repeat of this complete experiment yielded similar observations.

DISCUSSION

In earlier observations in which young or old mice were injected with tumor cells, we noted a slower growth rate, and a greater fibrous tissue component in the tumors of the old mice (Ershler *et al.*, 1984). To explain this apparent age-advantage we proposed that the presence of a greater fibrous component in the tumors of old animals reflected a containment mechanism which was more successful in the senescent host. We therefore examined in these experiments a functional assay of fibrosis, the wound healing response in young and old mice. We found that with primary intention wound healing older mice regained baseline skin strength more quickly than young and this was associated with

greater fibrosis demonstrated histologically. This suggestion is consistent with observations made in a similar experimental model in which an increase in breaking time of single tail tendon fibers was determined to correlate directly with aging in certain rodent species (Harrison *et al.*, 1978). It was therefore proposed that an exuberant fibrotic response to the transplanted tumor might, in part, explain the retarded growth in older animals. If this were true, inhibition of the fibrotic response would be expected to have a greater impact on tumor growth in the old animals. Extending this argument, if the exuberant fibrotic tumor containment were the *only* explanation for the observed age-related difference in tumor growth, then inhibition of fibrosis would be expected to reverse this age-advantage and there would be no difference in tumor growth, young versus old. With respect to these hypotheses we observed that inhibition of fibrogenesis had a striking effect on tumor growth in old mice, but no statistically significant effect in young mice. Tumor volume in the DHP treated old animals, however, was still significantly less than DHP treated young animals. We interpret these findings to indicate that a more prominent fibrotic response occurs in the older animals exposed to transplanted B16 tumor, and that this response is in part, but not totally responsible for the observed age-advantage with regard to local tumor containment. A mechanism or explanation for this more vigorous fibrotic response in the older animals may eventually be shown to be related to other local factors such as epidermal growth factors and capacity for angiogenesis.

In addition to the effect of a more prominent fibrous containment, other explanations for the apparent age-advantage in this model include endocrine, nutritional and immune factors which may favor more rapid growth in young animals. Additionally the host tissue response to any of a variety of angiogenesis or other growth factors may differ greatly with aging or the tissues themselves may be lacking in factors essential for optimal growth. Further investigation and elucidation of this age advantage may add useful information both to our understanding of the host factors that favor tumor growth and also our general understanding of the biology of aging.

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AGE- AND GENOTYPE-DEPENDENT DIFFERENCES IN CATECHOLAMINE CONCENTRATIONS IN THE PORCINE CAUDATE NUCLEUS

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(*Received* 26 *March* 1984)

Abstract—Catecholamine concentrations of the caudate nucleus were determined by radioenzymatic assay in stress-susceptible and stress-resistent pigs at five different ages. Dopamine concentrations increased with age up to the time of puberty and declined thereafter in both genotypes of pigs. Generally, epinephrine concentrations increased with increasing age in both genotypes of pigs. Norepinephrine concentrations remained relatively constant for all ages of pigs except the oldest pigs. For most age groups, dopamine concentrations were lower in pigs of stress-susceptible genotype than in stress-resistant genotype.

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INTRODUCTION

STRESS-SUSCEPTIBLE (SS) PIGS exhibit locomotor and neurochemical disturbances characteristic of certain basal ganglia disorders (Topel *et al.*, 1968). We have demonstrated catecholamine deficiencies in the caudate nucleus of pigs with the SS genotype (Altrogge *et al.*, 1980; Hallberg *et al.*, 1983). Initially, we observed lower amounts of dopamine, but not of epinephrine or norepinephrine, in 24-hour urine samples of SS pigs as compared with stress-resistant (SR) pigs. Subsequently, we found that five- to six-month-old pigs have lower dopamine concentrations, but not epinephrine or norepinephrine, in the caudate nucleus than do SR pigs (Altrogge *et al.*, 1980). In a follow-up study in which pigs were physically stressed before killing, we determined that the concentrations of dopamine, epinephrine, and norepinephrine were lower in the caudate nuclei and substantia nigrae of SS pigs than in the same structures of SR pigs (Hallberg *et al.*, 1983). In the present study, we examined the effects of age on catecholamine concentrations in the caudate nuclei of two genotypes of pigs.

MATERIALS AND METHODS

One hundred twenty one pigs ranging in age from six weeks to three and one-half years were obtained from the Iowa State University Swine Breeding Farm. Pigs were grouped according to five different ages to obtain ap-

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proximately equal numbers in each age group and to reflect different life stages of pigs. The first three groups represent rapidly growing animals (1 = <2 mos, 2 = >2–3 mos, 3 = >3–5 mos), whereas group 4 pigs were pubescent (>5–7 mos) and group 5 pigs were considered aged (1.5–3.5 yrs). Pigs were classified as either SS or SR by H-blood typing (Rasmusen and Christian, 1976), serum creatine phosphokinase activities (Rosalki, 1965; Sigma Chemical Co., 1973; Hallberg *et al.*, 1979), and halothane screening procedures (Christian, 1974). All pigs received a corn-soybean nonmedicated feed and water from weaning until death. All pigs were killed by electrical stunning and exsanguination between 0800 and 0900 hours. A craniotomy was performed on each pig, and the brain was removed from the skull within two minutes of death. After cutting the corpus callosum and reflecting the cerebral hemispheres laterally, the right and left caudate nuclei were dissected away from the laterally positioned internal capsule by a glass dissecting rod. The isolated caudate nucleus was immersed immediately in liquid nitrogen and subsequently packaged and stored at −80 °C. A radioenzymatic assay was used to determine the concentrations of dopamine, epinephrine, and norepinephrine in the porcine caudate nuclei (Passon and Peuler, 1973; Upjohn Diagnostics, 1978). Data were analyzed statistically by least-squares analysis of variance. The effects of genotype, age, and the interaction of age-by-genotype on dopamine, epinephrine, and norepinephrine concentrations in the caudate nuclei were included in the model. Means of significant effects only were separated by Fisher's Least Significant Difference (Snedecor and Cochran, 1967).

RESULTS

Mean concentrations of dopamine and epinephrine in the caudate nuclei of all pigs are presented in Table 1. Significant interactions noted between age and genotype of pig include the switch in rank of dopamine concentrations for groups 1 and 2. The magnitude of decline in dopamine concentrations following puberty (group 4 *vs* group 5) seems greater in SS pigs (37%) than in SR pigs (18%). An age-group breakdown of mean dopamine concentrations for both genotypes of pigs shows that, in the very young pigs (group 1), the SS pigs had greater dopamine concentrations than did the SR pigs. In all other age groups, however, SS pigs had from 20 to 40% less dopamine in the caudate

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TABLE 1. LEAST SQUARE MEANS OF CAUDATE NUCLEUS DOPAMINE AND EPINEPHRINE CONCENTRATIONS IN STRESS-SUSCEPTIBLE AND STRESS-RESISTANT PIGS OF DIFFERENT AGES.

	Dopamine		*Epinephrine*	
Age Group (mos or yrs)	*Stress-resistant*	*Stress-susceptible*	*Stress-resistant*	*Stress-susceptible*
	ng/g tissue			
1 (<2 mos)	2971 ± 737[a] (10)	4085 ± 601 (15)	146 ± 28[d] (10)	290 ± 43 (15)
2 (>2–3 mos)	6006 ± 548[b] (18)	3453 ± 672* (12)	347 ± 39 (18)	160 ± 48 (12)
3 (>3–5 mos)	7899 ± 622 (14)	5520 ± 564* (17)	349 ± 44 (14)	259 ± 40 (17)
4 (>5–7 mos)	8972 ± 601 (15)	7199 ± 737[c] (10)	327 ± 43 (15)	313 ± 53 (10)
5 (1.5–3.5 yrs)	7326 ± 1042 (5)	4613 ± 1042 (5)	468 ± 83 (4)	340 ± 83 (4)

Each value is a mean ± SEM. The number of pigs per group is given in parenthesis below each value.

*Stress-susceptible means are significantly different from stress-resistant means within an age group ($P < .05$).

[a]Group 1 stress-resistant mean is significantly different from the means of groups 2, 3, 4, and 5 ($P < .05$).

[b]Group 2 stress-resistant mean is significantly different from the mean of group 4 ($P < .05$).

[c]Group 4 stress-susceptible mean is significantly different from the means of groups 1, 2, and 5 ($P < .05$).

[d]Group 1 stress-resistant mean is significantly different from the means of groups 2, 3, 4, and 5 ($P < .05$).

nucleus than did SR pigs; for age groups 2 and 3, the difference in dopamine concentrations were significant ($P < .05$). With the exception of the first and second SS age groups, the mean dopamine concentrations increased with age up to the time of puberty and declined thereafter. Specifically, pubescent pigs had more than twice the dopamine concentration than did the youngest group of pigs when both genotypes were considered. The oldest animals of both genotypes (group 5) had dopamine concentrations that were 25% lower than those of pubescent animals. Within genotypes, SR pigs of group 1 had lower ($P < .05$) dopamine concentrations than did pigs in all other age groups. Additionally, there were lower ($P < .05$) dopamine concentrations in age group 2 than in age group 4 SR pigs. The SS pigs of group 4 had greater concentrations ($P < .05$) of dopamine than pigs in SS groups 1, 2, and 5.

Interactions between age and genotype also were observed for epinephrine concentrations in the caudate nucleus (Table 1). As was the case for dopamine concentrations, epinephrine concentrations for SS pig age groups 1 and 2 were reversed as compared with these values in groups 1 and 2 of SR pigs. Generally, concentrations of epinephrine, however, increased with age, with the greatest concentrations occurring in the oldest group of pigs. The youngest SR pigs (group 1) had significantly lower epinephrine concentrations than did the pigs of all other age groups. In contrast, group 2 of the SS pigs had the lowest epinephrine concentrations. The magnitude of increase of epinephrine concentrations with age was considerably different for SS pigs than for SR pigs. The oldest SS pigs had epinephrine concentrations that were similar to those of younger SR pig age groups 2, 3, and 4. Although epinephrine concentrations between genotypes of pigs were not different ($P > .05$), there was a trend for SS pigs to have less epinephrine in their caudate nuclei ($P < .12$). With the exception of the first age group, SS pigs consistently had lower concentrations of epinephrine in the caudate nucleus.

There were no age, genotypic, or age-by-genotype interaction effects ($P > .05$) on norepinephrine concentrations in the caudate nucleus. Concentrations of norepinephrine were relatively constant as age increased from 42 days (126 ng/g tissue) to 150 days (167 ng/g tissue), with the greatest concentration occurring at the latter age. In comparison with those of young pigs, approximately a two-fold decrease in norepinephrine concentration occurred in the oldest group of animals (71 ng/g tissue).

DISCUSSION

We have confirmed our previous findings of genetic differences in dopamine concentrations in the porcine caudate nucleus (Altrogge *et al.*, 1980; Hallberg *et al.*, 1983). Moreover, we have demonstrated an age-genotype interaction in dopamine concentrations. In combination with our earlier work, our findings are suggestive of a disturbance of the dopaminergic system of the basal ganglia of the SS pigs. Similar disturbances have been observed in different genotypes of mice (Ciaranello *et al.*, 1972; Ross *et al.*, 1976; Baker *et al.*, 1979; Reis *et al.*, 1979, 1981; Severson *et al.*, 1981), aged rodents and man (Finch, 1977, 1978; McGeer and McGeer, 1978; Randall, 1980; Severson and Finch, 1980; Osterburg *et al.*, 1981; Daszuta *et al.*, 1982; Trabucchi *et al.*, 1982), and in Parkinson's disease patients (Bernheimer *et al.*, 1973; Birkmayer *et al.*, 1975; Hornykiewicz, 1975; McGeer and McGeer, 1976). CBA/J mice have 20% lower striatal tyrosine hydroxylase activity, the rate-limiting enzyme of the biosynthesis of dopamine from tyrosine, than do Balb/cJ mice (Reis *et al.*, 1976; Reis *et al.*, 1979). In these same mouse genotypes, there

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are 25% fewer dopaminergic neurons in the substantia nigra of CBA/J mice and a 16% reduction in caudate nuclei cell number and volume as compared with Balb/cJ mice (Reis *et al.*, 1976; Reis *et al.*, 1979). Aging rats seem to have decreasing rates of dopamine systhesis, dopamine-sensitive adenyl cyclase activity and dopamine receptor activity in the caudate nucleus and other basal ganglia structures (Severson and Finch, 1980; Trabucchi *et al.*, 1982). The progressive dysfunction of the dopaminergic system in the rat and other animals is considered by many to be part of the normal aging process of the brain (Trabucchi *et al.*, 1982). Because of differential responses of dopamine concentrations with age in our two pig genotypes, the aging process of the SS pig evidently differs from that of the SR pig.

Clinically, Parkinson's patients have abnormally low amounts of dopamine in the caudate nucleus (Bernheimer *et al.*, 1973; Hornykiewicz, 1975; McGeer and McGeer, 1976). The dopamine deficiency in Parkinson's disease is thought to be because of a degeneration of either dopaminergic neurons in the substantia nigra or nigrostriatal pathway (Bernheimer *et al.*, 1973; Birkmayer *et al.*, 1975; Hornykiewicz, 1975). The tremors and rigidity seen in Parkinson's patients have been related to the death of nigral dopaminergic neurons and the consequential loss of striatal dopamine (Marsden *et al.*, 1975). It is not known whether the SS pigs have fewer dopaminergic neurons in the substantia nigra than do SR pigs, but our unpublished data indicates differences in striatal neuronal density of SS pigs compared with SR pigs. Additional investigations are needed to determine whether the lower dopamine concentrations in the caudate nucleus of SS pigs are because of fewer dopaminergic neurons or because of disturbances of dopamine metabolism. Nevertheless, the stress syndrome in pigs seems related to a disturbance of the dopaminergic system of the basal ganglia rather than to a dysfunction of either epinephrine or norepinephrine metabolism, because no significant genotypic difference in concentrations of these two catecholamine neurotransmitters in the caudate nuclei were observed in this study.

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AGING CHANGES IN THE HEART OF A POECILIID FISH, THE GUPPY *POECILIA RETICULATUS**

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(*Received* 4 *June* 1984)

Abstract—Histological surveys of the hearts of guppies *Poecilia reticulatus* throughout their lifespan showed no aging changes until the fish were three years old. In younger fishes the hearts were healthy, even in three individuals with "wasting" disease, where there was considerable loss of muscle throughout the body. There was a loss of muscle fibers in the ventricle in old fish, and deposition of collagen in the bulbus arteriosus. Guppies of three years and older also had a marked accumulation of melanomacrophages in the atrium. It seems unlikely that degeneration of the heart is involved in mortality of guppies in the wild.

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INTRODUCTION

FISH PROVIDE the best quantitative example among the vertebrates of the survival of a class under a variety of unusual and extreme environmental conditions. Such an ability demands functional plasticity in organ systems, one of the most important being the heart. Research has shown that the hearts of poikilotherms are better able to withstand or compensate for hypoxia, hypercapric acidoses, and stress-induced accelerations than are the hearts of homeotherms (Helle, 1983). Similarly, poikilotherms at their physiological temperatures are less affected by Ca^{2+}-deprivation-restitution treatment by comparison with the physiologically constrained hearts of warm-blooded vertebrates at 37°C (Langestrand *et al.*, 1983).

Fish hearts appear to be physiologically "spared" in their other functions. The reticuloendothelial system is a vital part of the defense system of fish, in which the heart plays a major role. Large populations of wandering and fixed macrophages in the peritoneal cavity, in the intermuscular spaces of the atrium of the heart, in the white pulp of the spleen, in periportal lymphoid areas of the kidney, and in the periportal tissue of the liver efficiently remove particulate matter from the blood (Mackmull and Michels, 1932; Roberts, 1975; Ellis *et al.*, 1976; Woodhead, 1981). The replete cardiac macrophages rapidly emigrate from the heart to other sites in the body, while in the spleen, liver, kidney and peritoneal cavity, the laden macrophages form aggregates, often in association with pre-existing melanomacrophage centers.

*Research done under the auspices of the U.S. Department of Energy.

In a series of experiments in which we gave several radiolabeled chemical carcinogens by intraperitoneal injection to Amazon mollies (*Poecilia formosa*) we noted that the cardiac macrophages were as intensely active in scavenging these compounds as they are with particulates. Within two hours after injection of dimethylbenz(a)anthracene (DMBA), label was present in the atrial macrophages and continued to accumulate there for the following 78 hours; radioactivity then declined at 102 hours and thereafter fell rapidly (Woodhead and Bornbusch, 1983). In the same way, radiolabeled *N*-methyl-*N*-nitrosourea (MNU) was taken up in large amounts by the atrial macrophages two hours after injections, and accumulated there in greater intensity than in the splenic or renal macrophages (Woodhead *et al.*, in press). Edelstein (1971) proposed that melanin, which has the ability to bind aromatic and cyclic compounds may selectively take up harmful chemicals from the circulation. This avidity with which the cardiac macrophages engulf particulates and clear chemical carcinogens from the blood stream might well compromise heart function and lead to local necrosis were the laden cells to form large aggregates *in situ,* as they do in other organs.

Cardiac failure does not appear to be a significant cause of death in fishes, although the condition is not unknown in wild and domesticated fish (Prior *et al.*, 1968), and may be associated with bacterial infections, including aeromonads and vibrios (Roberts, 1978). Degenerative changes in the arteries of migrating prespawning salmonids have also been described (Robertson *et al.*, 1961; Van Citters and Watson, 1968; Maneche *et al.*, 1972), but although the majority of fish exhibit vascular degeneration, the lesions are reversible, as shown by the reduction in their severity in postspawning survivors. Indeed, the lesions often have regressed completely as the fish start to return to their feeding grounds.

We have described aging changes in several organs of the guppy (Woodhead, 1979) and were interested in whether the heart of old fish develops lesions that might limit its functional ability. There is an occasional mention in the literature of aging changes in the heart: thus, Maneche *et al.* (1972) suggested that age may play a role in determining the extent of the vascular lesions in the spawning Atlantic salmon *Salmo salar.* Prior *et al.* (1968) also found an increase with age in the prevalence of calcified heart disease in brown trout *Salmo trutta,* and Roberts (1975) reported that the degree of the pigmentation of macrophages may increase with age.

Some years ago Dr. Alex Comfort generously made available to me a collection of histological material from guppies *Poecilia reticulatus* that he had kept into extreme old age under excellent conditions, and had sampled at frequent intervals. The fish had not been treated experimentally in any way, and were designated "normal growers" (Comfort, 1960). These histological preparations provided an opportunity to conduct a survey of the histological changes in the heart throughout their lifespan.

MATERIALS AND METHODS

The guppies came from a laboratory population maintained into old age by Comfort (1960). The survey was based upon serial sections of 20 females and 20 male fish, ranging in age from 14 days to 61 months. The sections had been cut at 8 μm and the majority of slides stained with haematoxylin and eosin; sections from five fish had been stained with Mallory's trichrome stain for connective tissue. A qualitative description of the state of the atrium, ventricle and bulbus arteriosus was made for each fish, and any abnormality was noted. The area of cardiac muscle in the ventricle was measured by tracing an outline of the organ on to paper of standard thickness, then cutting out the lacunae, and weighing the cut-out parts; this value was compared with the weight of the entire ventricle. A second estimate was obtained by projecting an image of the section on to a television screen, and marking off the extent of muscle tissue along a predetermined line. I also counted the number of melanin-containing cells in five random fields of the atrium of young and old fish, adopting Robert's (1975) definition of melanin to include all pigments ranging from yellow to dark-brown.

As in previous studies, the age of two years was adopted as a time in the life-cycle at which we could make an arbitrary but convenient distinction between "young" and "old" individuals (Woodhead and Ellett, 1967).

RESULTS

There was no obvious deterioration in the heart of any guppy of less than two years of age; indeed, no lesions were apparent until the fish had attained three years (1098 days). Even so, three of the fish in the series that were older had hearts without lesions or loss of musculature. Only one fish, a female aged 1859 days, had a definitive lesions in the bulbus arteriosus that resembled an atheromatous plaque. There were no sex differences in the time of onset of the aging changes in the heart, nor in the extent or type of deterioration.

Amongst the young fish were three individuals with "wasting" disease, in which there was extensive loss of the body musculature; in none of these individuals was the cardiac muscle affected.

Bulbus Arteriosus

The bulbus arteriosus is a distinct region of cardiac origin lying between the heart and the ventral aorta. It is a highly elastic organ, its rebound maintaining and accounting for 25% of the blood flow along the aorta during ventricular diastole (Priede, 1976). An outer, relatively inextensible layer of connective tissue and collagen (the so-called "adventitia") in which there are small arteries, veins and lymphatics covers the "media," a compact layer of smooth muscle and elastic tissue. This layer forms the bulk of the wall of the bulbus arteriosus. In our sections stained with haematoxylyin and eosin, the "media" appeared to be composed of muscle tissue only. In young fish near to the junction with the ventral aorta, the "media" was thin, with regular, well-defined circumferential muscle layers, staining a deep purple. A single layer of endothelial cells, the intima, lined the lumen of the vessel. Towards the heart, the media thickened, and bundles of muscle fibers running radially and longitudinally were also apparent (Plate 1*a*).

In fish aged three years or more, the muscle layers of the media had increased in thickness, particularly the circumferential bands. The muscle fibers were accentric and ill-defined, being widely separated by pale-staining collagen deposits. In the oldest fishes in the series there were rather few muscle fibers altogether and collagen, which showed considerable hyaline degeneration, had taken their place (Plate 1*b*). In some older individuals there were areas of vacuolation containing traces of cellular membranes lying immediately beneath the endothelium; these spaces and debris appeared to represent the remains of fat cells that had been dissolved during fixation of the material. One fish showed heavy deposits of calcium in the "media." In most old fish the intima remained intact, with only an occasional area of hypertrophy or hyperplasia.

One fish had a lesion that resembled an atheromatous plaque. The wall of the bulbus had ruptured, releasing a pink-staining hyaline material into the vessel lumen, and to the exterior. At the site of rupture the walls of the bulbus were ragged and irregular, with small deposits of calcium in the media. The lesion was associated with a large external area of thrombosis.

Ventricle

In the guppy the ventricle is composed of a meshwork of spongy avascular traveculae, the blood flow in the organ being restricted to the lacunae. In young guppies the muscular traveculae were broad and closely intermeshed, enclosing numerous small lacunae (Plate 2*a*).

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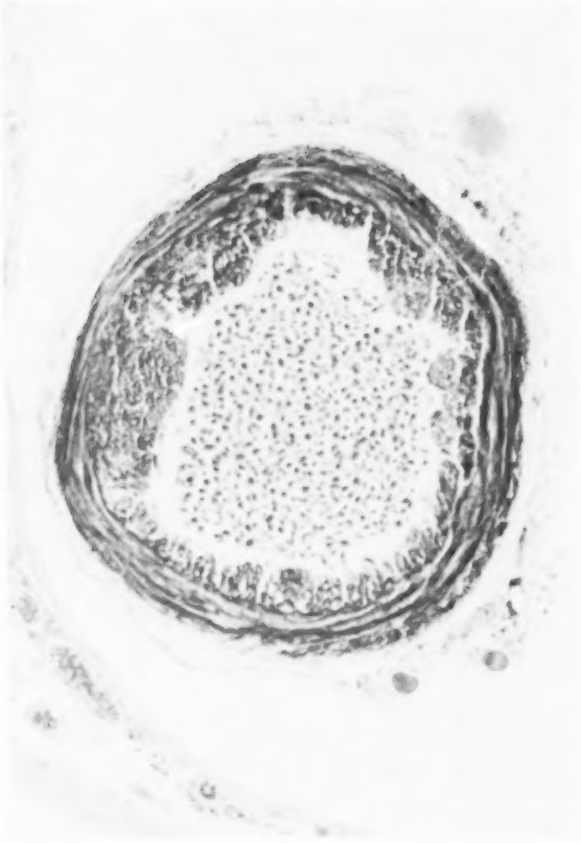

PLATE 1*a*. Section through the bulbus arteriosus of a young guppy aged 187 days showing the well-defined layers of circumferential, radial, and longitudinal muscle fibers. ×300.

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PLATE 1*b*. The bulbus arteriosus of a fish of 1900 days with ill-defined muscle bands, loss of muscle and its replacement by collagen. ×120.

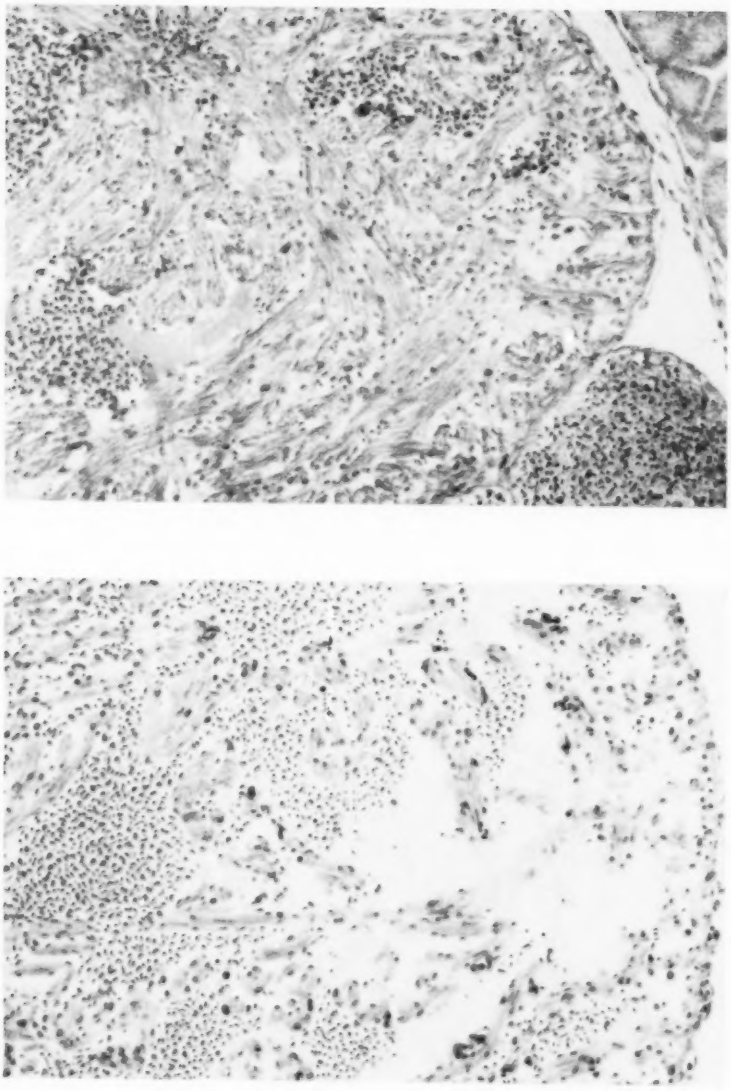

PLATE 2. Transverse section through the ventricle of a (a) young, (b) old guppy illustrating the loss of muscle fibers in the heart of the old fish. ×250.

In fish older than three years there had been a marked loss and thinning of the muscle fibers, and correspondingly the lacunae were larger and the entire organ flaccid (Plate 2*b*; Table 1). Older fish showed areas of vacuolation and necrosis of muscle fibrils.

No yellow-golden nor dark brown melanocytes were seen in the ventricles of young guppies. In older fish, however, melanocytes were often present, usually singly, but occasionally there were larger groups occurring on the wall of the ventricle adjacent to the atrium (Plate 3*b*). These cells had accumulated much pigment, and were dark brown to black in color.

Atrium

The most noteable difference between the atrium of young fish and that of old individuals was the large increase in the numbers of yellow-golden and dark brown melanocytes in the organ. In young fish there were a few pigmented cells associated with the muscle fibers, but melanocytes lined every muscle fiber in individuals older than three years, and there were also small groups of cells here and there (Plate 3*a, b;* Table 2). By contrast, in guppies of about one year of age, there was already a marked accumulation of melanocytes in the melanomacrophage centers of the kidney (Woodhead *et al.*, 1983).

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TABLE 1. COMPARISON OF THE EXTENT OF MUSCLE FIBER IN THE VENTRICLE OF FOUR FISH (A) YOUNGER THAN 2 YEARS, (B) OLDER THAN THREE YEARS. COLUMN A IS THE VALUE OBTAINED BY MEASUREMENT OF AREA, COLUMN B WAS OBTAINED BY LINE PROJECTION.

	% muscle in ventricle			*% muscle in ventricle*	
"Young"	*A*	*B*	*"Old"*	*A*	*B*
1	62	59	1	30	32
2	64	62	2	48	47
3	56	55	3	45	46
4	80	72	4	36	37

TABLE 2. THE NUMBERS OF MELANOCYTES IN STANDARD FIELDS OF THE ATRIUM OF FOUR FISH (A) YOUNGER THAN 2 YEARS, (B) OLDER THAN THREE YEARS. THE VALUES GIVEN ARE THE MEANS FROM THE FIVE READINGS.

"Young"	*Number of melanocytes*	*"Old"*	*Number of melanocytes*
1	20	1	143
2	12	2	218
3	15	3	107
4	7	4	98

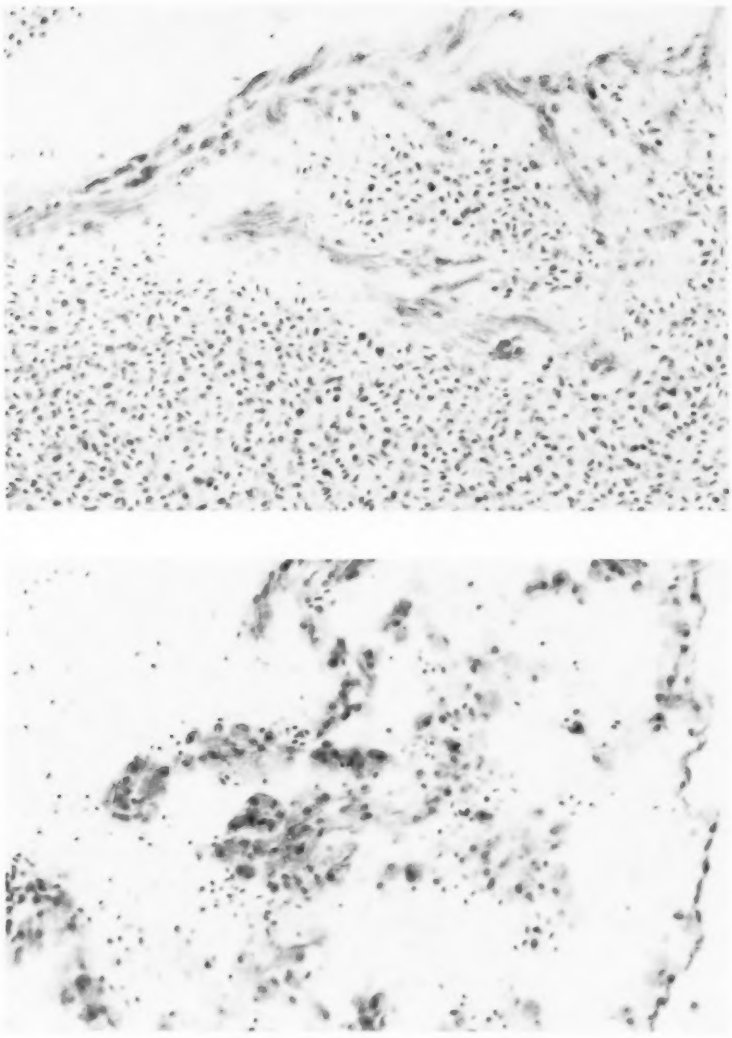

PLATE 3. Comparison of the accumulation of macrophages along the atrial fibers of (a) young, and (b) old guppies. ×400.

DISCUSSION

Guppies appear to enjoy a considerable degree of protection from heart disease. Degenerative changes, albeit minor at first but progressing with time, are discernible from the age of two years in many organs (Woodhead, 1979). However, the heart showed no aging features until the guppies had reached three years of age, a longevity that they are

unlikely to attain in the wild state. There was no loss of cardiac muscle until this late age, and only one fish from the series had a major lesion in its heart (bulbus arteriosus). Three "young" fish of under two years suffered extensive wasting disease that affected all parts of the body musculature; their hearts, however, were unaffected, and were firm and healthy.

Macrophages are essential for normal scavenging of particulates and chemicals, and the cardiac macrophages contribute significantly to this defense system. Because of their activity, or their particularly vulnerable site in the body, atrial macrophages quickly accumulate foreign materials from the circulation and then emigrate from the heart. The process of emigration (as defined by the number of macrophages remaining in the heart) does not seem to diminish until the fish are older than three years, after which these cells accumulate in large numbers in the atrium. However, even then the ventricle is comparatively free from macrophages. Our data therefore indicate that aging changes in the heart of the guppy are likely to make only a minimal contribution to natural mortality.

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THE EFFECT OF DIETARY METHIONINE ON THE COPPER CONTENT OF TISSUES AND SURVIVAL OF YOUNG AND OLD MICE

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Abstract—We tested the possibility that methionine might improve the lifespan of male C57BL/6J mice, based upon the ability of methionine to chelate copper. Old mice given 0.05M methionine in their drinking water for 42 days had lower brain copper concentrations ($p < 0.05$). The decrease in liver, kidney, and heart copper was not significant when compared to unsupplemented controls. The lifespan of old mice was unchanged by feeding 0.05M methionine. Young mice, however, experienced a 16.9% decrease in their average lifespan and a decreased maximum lifespan when given supplemental methionine. We conclude that dietary supplements of methionine may be useful for removing copper from the brain but they also can increase the rate of senescence in mice.

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INTRODUCTION

METHIONINE IS an essential amino acid needed for normal metabolic function. Lippman (1980, 1981) has suggested that methionine may also function as an inhibitor of senescence based upon its ability to inhibit superoxide radical production from metabolically-active human mitochondria.

Other observations suggest a protective role for methionine in senescence. Amyloid formation is inhibited by methionine (Kornejewa, 1970). A combination of vitamin E and methionine induces glutathione peroxidase activity (Kruhlykova and Shtutman, 1976). Methionine decreases lipid peroxidation induced by carbon tetrachloride (Hafeman and Hoekstra, 1977). The eye lenses of old humans show oxidation of methionine in membrane fractions with extensive oxidation occurring in cataracts (Garner and Spector, 1980). Methionine protects rats from carcinogenesis induced by aflatoxin B_1, N-2-fluorenylacetamide, 1,2-dimethylhydrazine (Rogers, *et al.*, 1980) and ethionine (Farber and Ichinose, 1958). Nucleolar volume and polymerase activity in the liver may depend upon dietary methionine (Bailey, *et al.*, 1976).

A diet low in methionine leads to elevated cholesterol levels and atherosclerosis in rats, mice and monkeys (Mann, *et al.*, 1953; Fillios and Mann, 1954; Mann, 1961; Clandinin and Yamashiro, 1980) but has no effect on pigs (Hill, *et al.*, 1971).

In bacteria, certain methionine auxotrophs of *Escherichia coli* lose their colony-forming ability when deprived of methionine, resulting in "methionineless death" (Breit-

man, *et al.*, 1971). A decay of messenger RNA "with a survival curve similar to that of an aging process," also occurs during methionine starvation (Silengo, 1973).

One possible explanation for the favorable effects of methionine may be its ability to chelate copper ions with an equilibrium constant of 14.75 (Chaberek and Martell, 1959). It is known that dietary methionine counteracts the elevated levels of copper in blood plasma and livers of chickens fed diets containing high concentrations of copper (Jensen and Maurice, 1979). This suggested to us that dietary methionine might influence the increase in brain copper known to occur in mice and humans with aging (Schroeder, *et al.*, 1966; Massie, *et al.*, 1979a). Older rats absorb methionine at a slower rate (Pénzes, *et al.*, 1968), and it disappears more rapidly from the tissues of old rats than from young (Barrows and Roeder, 1961). Thus, the increase with aging in both brain and serum copper in mice and humans (Harman, 1965; Herring, *et al.*, 1960; Yunice, *et al.*, 1974; Massie, *et al.*, 1979b) could be due to insufficient dietary methionine. Harman (1965) has proposed that elevated copper levels may accelerate aging by acting as a catalyst for the production of free radicals. Higher dietary levels of methionine should, therefore, improve survival if they are able to lower tissue copper concentrations.

Here we report that high dietary methionine lowers brain copper concentrations but does not increase lifespan.

MATERIALS AND METHODS

Biological sample and diet

Male C57BL/6J mice obtained from Jackson Labs., Bar Harbor, Maine, were used for all experiments. Mice were purchased at one month of age and introduced into our colony. Purina laboratory chow (which contained 18 ppm copper in the ash) and tap water were given *ad libitum* to the aging colony. According to the label the Purina laboratory chow contained 23.4% protein, 4.5% fat and 0.43% methionine. Animals were kept at 22°C and lights were on 12 hours and off 12 hours.

Survival studies

Animals were removed from the aging colony at 42 and 581 days of age. Mice were placed 7-8 per cage in plastic cages with stainless steel tops. Corncob bedding and distilled water bottles were changed weekly. L-methionine (Sigma or Nutritional Biochemical Co.) was added to the drinking water for the experimental groups. A 0.05M methionine solution was prepared every 3 weeks and stored at 4°C. Mice were given fresh solution bottles weekly. Cages were monitored daily for deaths. Mice were weighed every two weeks until 150 days of age and thereafter monthly. Fighters or injured animals were removed from the group and placed in separate cages. Whenever possible fighters were removed from the experiment during the first few weeks. All animals were allowed to eat Purina laboratory chow pellets without restriction.

Data analysis

Student's *t*-test was used to establish significant differences between groups for both metal content and average survival times.

Metal determinations

Mice were sacrificed between 9 a.m. and 11 a.m. (Eastern Standard Time) in order to avoid possible diurnal changes. Organs were isolated and perfused with 0.1M HEPES buffer (pH 7.8). Single organs were then placed on acid-washed microscope slides and dried overnight in an oven at 88°C. We found that longer drying times did not decrease organ weights. Whole organs were digested in ULTREX HNO_3 (J.T. Baker Chemical Co.). Acid digestion was allowed to proceed for 7 days at room temperature. The thin fat layer formed on top of the liver samples was removed by aspiration. Some liver copper may have been lost by this procedure, but it was probably less than 1% of the total. Samples were analyzed on a Varian 1250 atomic absorption spectrophotometer with carbon-rod atomizer Model 90. Both young and old organ samples were checked by the method of standard additions for possible age-related interference with copper detection. None was found under our conditions.

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TABLE 1. COPPER CONTENT OF ORGANS FROM C57BL/6J MALE MICE FED 0.05M METHIONINE IN THEIR DRINKING WATER FOR 42 DAYS BEGINNING AT 538 DAYS OF AGE.

Organ	*Copper Content ng/mg dry wt.*[1]	*Number*	*Significance*
liver, control	12.04 ± 1.26	7	n.s.[2]
liver, fed	11.53 ± 1.37	6	
kidney, control	17.18 ± 1.00	7	n.s.
kidney, fed	16.72 ± 0.95	6	
brain, control	16.29 ± 0.77	7	$p < 0.05$
brain, fed	14.42 ± 1.72	6	
heart, control	21.15 ± 2.36	7	n.s.
heart, fed	18.81 ± 3.74	6	

[1]Data are averages ± standard deviation.
[2]n.s. indicates not significant ($p > 0.05$).

RESULTS

In order to find effective chelating compounds we routinely fed various compounds to old mice for 42 days. Feeding 538 day old mice 0.05M methionine for 42 days failed to significantly change the copper content of liver, kidney or heart (Table 1). The copper content of brain, however, was reduced from 16.3ng to 14.4ng/mg dry wt., with a degree of certainty of greater than 95%.

The lowering by methionine of brain copper concentrations to the levels found in young mice (Massie, *et al.*, 1979a) suggested that feeding excess dietary methionine to old mice might increase longevity. Feeding 0.05M methionine in the drinking water beginning at 581 days of age did result in greater stabilization of total body weight (Figure 1) but it failed to prolong the average lifespan (Figure 2). The average lifespan for the methionine group was 757 days and for the control 755 days (Table 2). It should be noted that the control group for this experiment was unusual. The weight versus age curve declined at a faster than normal rate (Figure 1) and the 755 day value for the average survival time was less than the 900 day value which we normally find. The control group also showed two abrupt drops in survival just prior to and after 800 days of age (Figure 2). The methionine-supplemented group, in contrast, showed an even decline with age. It is probable that the control group contained one or more fighters which we failed to recognize. Such an individual could cause early weight loss and premature death for his cage companions. The two abrupt declines in survival before and after 800 days of age in the control group (Figure 2) suggest that this is a likely possibility. Therefore, the survival curve for the control group should be regarded with suspicion. In view of this, methionine may in fact be toxic to old mice when compared to the expected survival curves.

When young (42 day old) mice were given 0.05M methionine for life, the body weight versus age curve showed essentially no difference between the control and the methionine group (Figure 3). Surprisingly, the average lifespan of the methionine group decreased by 16.9% ($p < 0.05$) (Table 2). Even the maximum lifespan decreased in this experiment (Figure 4). Since the weight versus age curves were essentially identical it is unlikely that differential food intake was responsible for the increased aging of the methionine group.

Our mice consumed about 4ml of liquid per day. The daily intake of methionine from

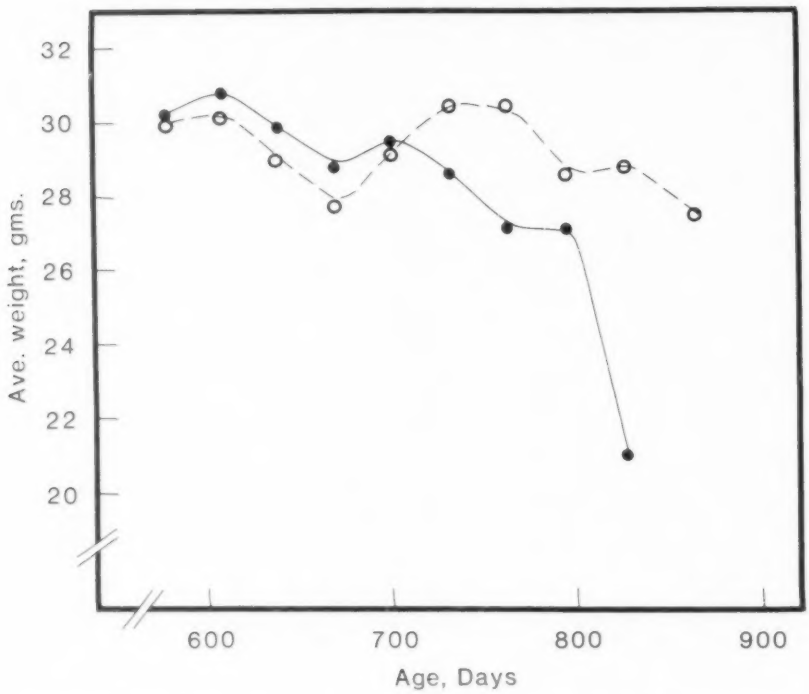

FIG. 1. Average weight in grams versus age for control (——) mice and mice fed 0.05M methionine (----) in their drinking water for the remainder of their life, beginning at 581 days of age.

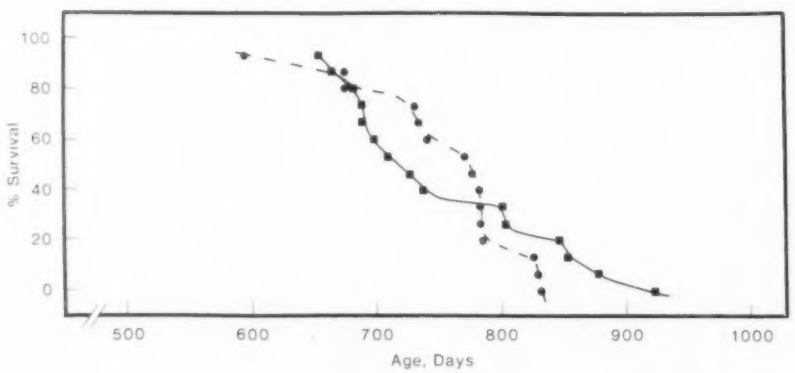

FIG. 2. Survival curves for control (●) and mice receiving 0.05M methionine (■) in their drinking water for the remainder of their life, beginning at 581 days of age.

TABLE 2. CHANGES IN LIFESPAN FROM CONTINUOUS FEEDING OF METHIONINE.

Age began, Days	*Methionine, M*	*Average life span, Days*[1]	*Number of Animals*	*% Change*	*P Value*
42	control	921 ± 109	8	–	
42	0.05	766 ± 113	8	−16.9	$0.01 < p < 0.05$
581	control	755 ± 65.5	15	–	
581	0.05	757 ± 86.4	15	0	$p > 0.05$

[1]Data are averages ± standard deviation.

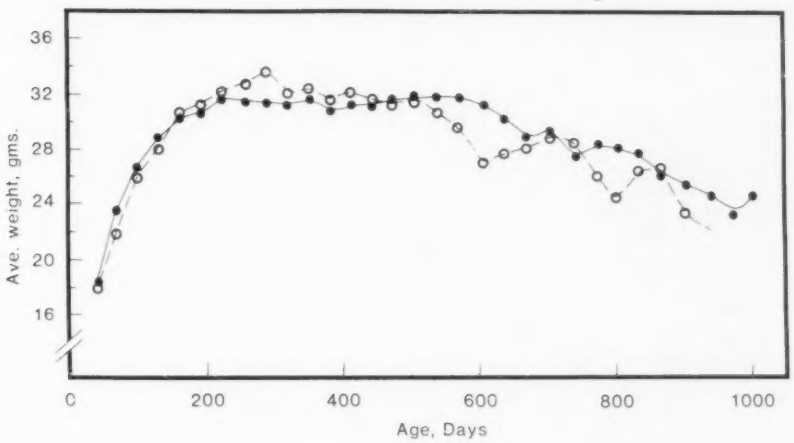

FIG. 3. Average weight in grams versus age for control (——) mice and mice fed 0.05M methionine (---) in their drinking water for life, beginning at 42 days of age.

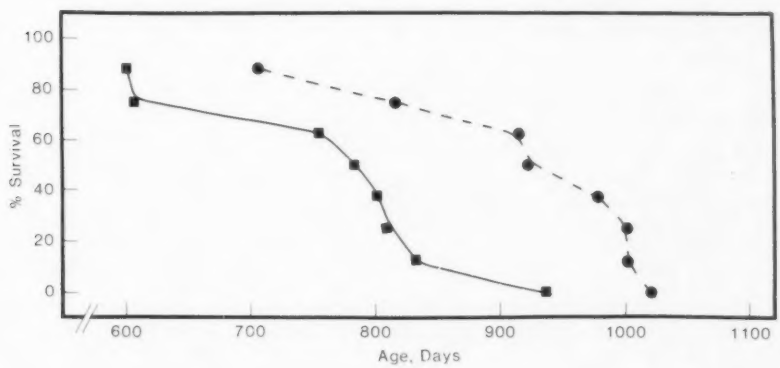

FIG. 4. Survival curves for control (●) and mice receiving 0.05M methionine (■) in their drinking water for life, beginning at 42 days of age.

the drinking water was thus 29.8mg/day. The total solid food consumed was about 5000mg/day and contained 0.43% methionine. Thus, methionine represented approximately 1.03% of the total solid intake. Assuming an average weight of 30gm for our mice, the total intake of methionine for supplemented mice was 1.67gm methionine/kg body weight/day or 11.2 millimoles methionine/kg/day.

In conclusion, dietary methionine was found to lower the copper levels in the brains of old mice but the average lifespan was unchanged. In contrast, feeding methionine to young mice shortened their lifespan. These results indicate that dietary methionine can shorten instead of lengthen the lifespan.

DISCUSSION

Harter and Baker (1978) observed a decreased rate of weight gain, lower blood hemoglobin and increased spleen iron levels for chicks fed 1% or more of dietary methionine. At 0.50% methionine there was little change from the control chicks. Our body weight versus age curves (Figures 1 and 3) indicate an absence of weight loss for the methionine-fed mice. It, therefore, seems unlikely that we used a methionine concentration where overt toxicity was a problem. It is clear, however, that methionine shortened lifespan in our experiments with young mice. Identification of the mode of action of methionine-induced reduction of longevity might lead to a better understanding of senescence. One possible mechanism could be the methionine-induced nuclear and nucleolar lesions reported by Shinozuka *et al.* (1971).

The amount of methionine given in this experiment was not large when compared to the methionine content of many food proteins. Milk protein from cows, for example, contains 2.6% methionine and egg albumen 4.6% methionine. Bird (1978) has described methionine as the most toxic of the nutritionally important amino acids. Thus, while rats can consume 1.5% methionine with no apparent decrease in the rate of growth (Benevenga and Harper, 1967), guinea pigs, rabbits and man are less tolerant of methionine (Hardwick, *et al.*, 1970). Our mice consumed a total of 11.2 millimoles methionine/kg body weight/day. A dose of 10 millimoles/kg/day results in inanition and death within 65 hrs for guinea pigs. A single intravenous dose of 2.8 millimoles/kg of methionine to humans produces nausea, vomiting, hypotension, tachycardia, fever, disorientation and liver disfunction (Floyd, *et al.*, 1966). It isn't clear why species differences are so great. Several proposals have been made, but none fully explains why methionine should be so toxic (Bird, 1978). Our results suggest that methionine may be changing copper metabolism, especially in the brain. The use of methionine as a means for slowing senescence is not supported by our data. In fact, diets high in methionine may actually accelerate senescence especially in those species showing greater sensitivity to methionine such as man.

Our results showing a slight but significant removal of brain copper with methionine suggest that methionine might be effective in the treatment of certain disorders of copper metabolism such as Wilson's disease where copper is known to accumulate in the brain.

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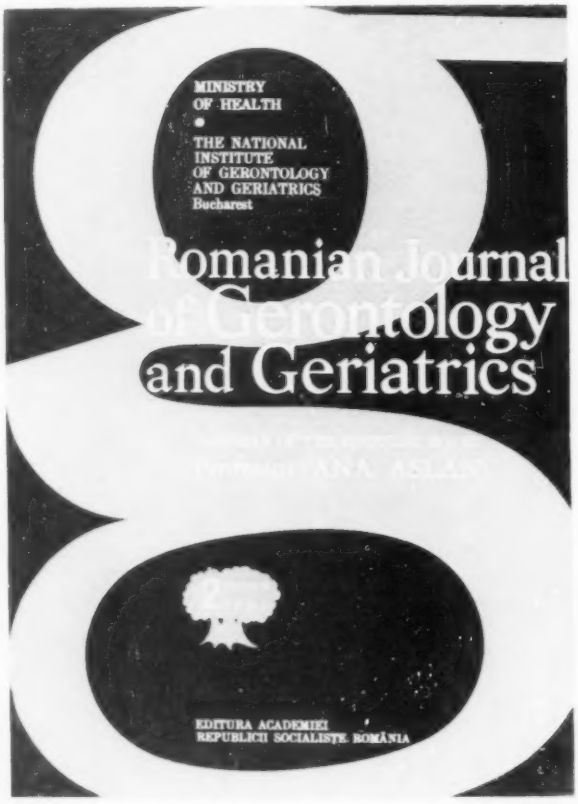

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LETTER TO THE EDITOR

We are requesting a correction of a misrepresentation or misstatement of our data which were referenced in a recent manuscript appearing in the JOURNAL (Baird, 1983, *Exp. Geront.* **18,** 47).

Baird has recently published data which suggest that (1) the well-documented increase in the duration of drug responses in aged animals is not universal and (2) the data obtained from "pooled" tissue extracts for the *in vitro* analysis of hepatic monooxygenase activities tend to mask such interindividual variability. While we do not disagree with Baird, we do object to his referring to studies performed in our laboratory as employing "pooled extracts of hepatic microsomal membranes" for analysis of microsomal mixed function oxidase activities. In this case the author referred to a review paper and not the original publications (Schmucker and Wang, 1980, *Proc. Soc. Exptl. Biol. Med.* **165,** 178). At no time have we ever employed "pooled" tissue samples to evaluate liver drug-metabolizing enzyme parameters in aging rats, but have always used groups of individual animals and reported the mean values and the appropriate statistical and probability values. In our opinion, Baird is insufficiently familiar with the studies from our laboratory to use these data in this context—a suspicion which is supported by the marked misspelling of the senior author's name.

Again, Baird uses this review paper as an example of comparing data from sexually immature rats with those from mature animals and suggesting that the changes in liver enzyme activities are a result of aging rather than shifts in endogenous hormone levels. In fact, we have never suggested in that review paper or in the original papers that the changes observed during maturation resulted from anything other than such factors, i.e. hormones. Lastly, the author ignores several subsequent papers from our laboratory which are particularly germane to his argument in that they employ young adult rats (3 months of age) (Schmucker and Wang, 1982, *Age* **5,** 105; 1983, *Mech. Aging Develop.* **21,** 137).

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AMERICAN GASTROENTEROLOGICAL ASSOCIATION COUNCIL ON AGING ESTABLISHES TISSUE EXCHANGE PROGRAM

The Council on Aging of the American Gastroenterological Association, in cooperation with the National Institute on Aging, has established a voluntary tissue exchange program for investigators interested in the aging process. The program will permit researchers to obtain valuable tissue and body fluid samples from animal models being used in aging studies. Information and an updated list of the participants in the program may be obtained from D.L. Schmucker, Cell Biology & Aging Section (151E), Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121.

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